Physiological Zoölogy

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Physiological Zoölogy

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PROTOPLASMIC REORGANIZATION IN URONYCHIA UNCINATA, N. SP., DURING BINARY FISSION AND REGENERATION

(Fifteen figures)

C. V. TAYLOR

Biological Laboratory, Stanford University, California

STEIN (1859) showed that during binary fission in various ciliates all motor organelles of the mother organism were completely resorbed and simultaneously a wholly new set emerged in each of the resulting daughters. This important discovery received relatively little attention until Wallengren (1900, 1901) published the results of his classical studies which confirmed and considerably extended Stein's observations. A critical review of the scanty literature is contained in these excellent papers by Wallengren.

It should here be noted also that Maupas (1889) briefly described the outgrowth of new cirri and disappearance of the old in conjugating pairs of *Euplotes patella*. Engelmann (1862) had mentioned a similar procedure for conjugating individuals of *E. charon*.

Furthermore, it has long been known, since Stein's (1854) earliest record of the fact for the hypotrich *Epiclintes*, that a disappearance of the ciliary apparatus occurs during encystment. Bütschli (1887–89) gives a critical discussion of later studies on this resorption process during encystment.

In a report made at the Cincinnati meeting of the American Society of Zoölogists (Taylor, 1923*a*), it was shown that regeneration

of micronucleate pieces in *Euplotes patella* involves profound protoplasmic changes which are altogether comparable with those accompanying its binary fission. The cirri and membranelles in the regenerating piece are entirely resorbed and a new set of these organelles appears in the reformed individual. Further experiments (unpublished data) on that organism gave confirmatory results. Similar changes in regenerating *Stylonychia* have recently been described by Dembowska (1925). Here, as in *Euplotes*, redifferentiation of a new set occurred in each regenerating piece. This investigator showed further, however, that these reorganizing changes resulted only when some one or more of the locomotor organs were affected in the operation.

Were it demonstrated that such deep-seated protoplasmic changes are characteristic for regeneration as they are for binary fission, and possibly for conjugation and encystment in ciliates, we should thereby attain knowledge of a common process which, it would seem, might very well come to rank among the fundamental properties of living matter. To this end, experimental studies were undertaken on the hypotrichous ciliate *Uronychia uncinata*, n. sp., following careful observations on its binary fission. Its giant posterior cirri and other prominent organelles afforded excellent means for an accurate analysis of structural changes that might occur during regeneration.

METHOD AND MATERIAL

Improved methods of micromanipulation have become an exceedingly useful, even indispensable, aid in modern experimental studies on living matter. The patent fact that protoplasm is organized into microscopic and often highly differentiated units called cells, makes necessary in its study the use of refined and accurately controllable apparatus, for the hand no less than for the eye. Accordingly, in this investigation utmost care has been given to the methods and equipment employed. The results, therefore, are regarded as altogether dependable. Thoroughly clean glassware, including pipettes, stender dishes, cover slips, the moist chamber, and quartz needles (sterilized in 95 per cent alcohol), were used throughout each operation. The quartz needles were operated within the moist chamber by means of a Taylor (1925) micromanipulator.

Each organism to be operated upon was first transferred to and washed in boiled sea-water. After the operation, the resulting pieces of the organism were transferred by means of a sterile mouth-pipette to a 1-inch stender dish containing boiled sea-water. The stender dishes were kept in moist chambers wherein the temperature varied between 15° and 17° C. The percentage of regenerating pieces was accordingly high (about 98 per cent) and, it is believed, entirely normal. During the process of regeneration, as occasion required, the piece was transferred to a large hanging drop of sterile sea-water, which was inverted and sealed by vaseline on a hanging- drop slide and studied under a monobjective binocular microscope with a 4 mm. objective and ×15 oculars.

Examination of the living organism during binary fission was likewise made in a hanging drop with the same magnification.

Fixed and stained material was prepared in the usual way. Killing reagents included Bouin's, Schaudinn's, and Benda's. Acetic acid was omitted owing to its tendency to cause the cirri and membranelles to break up into their component cilia. The modified Benda's reagent proved the most satisfactory for fixing the external organelles. Schaudinn's (without acetic) was best for study of the nuclei. For staining, Delafield's and the iron-haematoxylin methods were employed. The former stains beautifully the external organelles, but iron haematoxylin was a better nuclear dye. Counterstains were not used.

At least two species of Uronychia occur in Monterey Bay near the Hopkins Marine Station. Both are probably new species. The larger, which is more than twice the size of the smaller species, was found in considerable abundance in one of the aquaria where it has been known to occur for at least two years. It was used exclusively in these experiments and is herein described as a new species, to which has been assigned the name *U. uncinata*, for reasons given in later paragraphs.

THE LIVING ORGANISM

In both structure and behavior, *Uronychia* (Stein, 1865) is perhaps the most distinctive of the hypotrichous ciliates. Its conspicuous earmark—whence its name—is the group of nine giant cirri which project backward from fairly deep concavities in the posterior

end (Fig. 1). There are three such cavities and as many separate groups of the giant organelles. Cirruslike membranelles which extend anteriorly from the dorsal base of the oral lip, and prominent

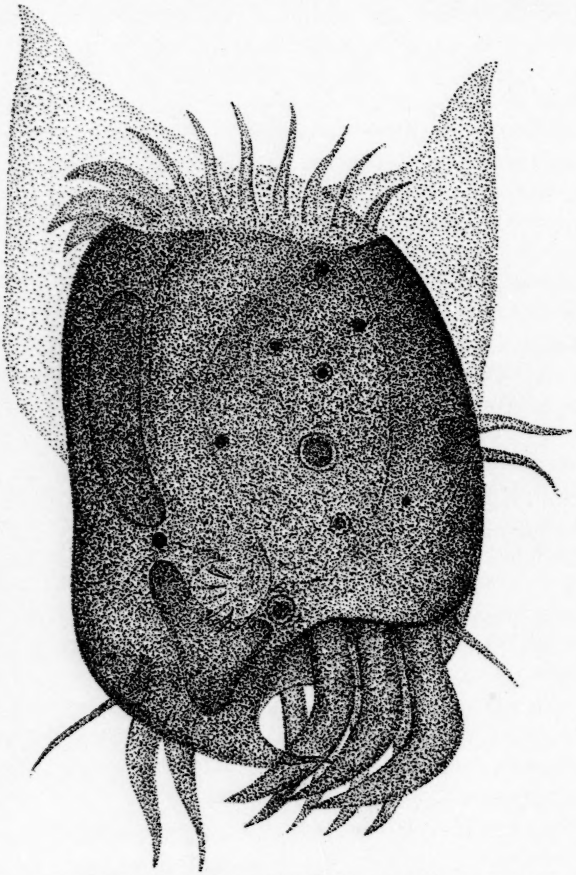


FIG. 1.—Camera-lucida drawing of a living, narcotized *Uronychia uncinata*. ×1,050.

undulating membranes that border a rather spacious cytostome are other external structures characteristic of the *Uronychia*. Young (1922) gives a fairly adequate diagnosis of the genus, to which refer-

ence may be made, so that further description here will have to do with this new species *U. uncinata.*

A group of three markedly sickle-shaped giant cirri, relatively on the right, extend from an arched cavity, postero-dorsal in origin and position. These are the largest of the nine, and are the largest cirri I have thus far seen in any of the hypotrichs. Located ventral to them is a second group of four more slender and less curved cirri, separated from the former by a thin shelf. On the left posterior is the third group of cirri, two in number and ventral in origin. These taper and curve gracefully and, as shown later, are by origin a double cirrus. As described under the caption "Binary Fission," all nine cirri tend to become more and more fimbriated as the individual approaches the period of fission.

A unique structural feature of body form should be noted here, to be referred to hereinafter as the "uncinus," for which this species receives its name. This singular acuminate hooklike structure marks an extension of the left posterior extremity of the body which curves mediad with its tip deflected outward. As indicated under the caption "Binary Fission," the uncinus apparently owes its origin in part to an attenuation of the remnant which for some time holds together the new daughter individuals (Fig. 5, p. 11).

The left pair of giant cirri originates ventral to the base of the uncinus.

Four much smaller, more slender ventral cirri complete the total number of thirteen of these external motor organelles. One of the four, quite long and slender, is situated somewhat anterior to the bases of the left giant pair. Another of the four originates near the outermost of the ventral group of four giant cirri. The remaining two ventral cirri, as shown in Figure 1, are located on the right side considerably anterior to the four giant cirri.

There are ten membranelles. These originate dorsal to and near the base of a prominent (in this species) oral lip (Fig. 1) over which they project anteriorly. Seven of the ten membranelles are essentially cirri. They are apparently quite comparable with the cirrus-like membranelles which Wallengren (1901), Calkins (1911), and Young (1922) have described for other species of this genus. The left-most three are more typically membranelles. These two types of

motor organelles should rightly be regarded as structurally transitional between the true cirri and membranelles (Taylor, 1920) which are common in the Hypotricha.

The prominent oral lip marks the anterior dorsal extension of the spacious cytostome. The latter in general outline is an elongated triangle with its apex directed posteriorly (Fig. 1). Extending along either side of the cytostome is a conspicuous wide undulating membrane. Under the base of the oral lip these right and left membranes converge gradually toward the median axis where they become contiguous. In some specimens fixed by means of the modified Benda's reagent, the membranes appear to be continuous at that point. The left undulating membrane is sometimes divided into an anterior and posterior part. The latter portion bounds the left side of a spheroidal cytopharynx which represents a sinistral outpocketing of the cytostomal apex. This cytopharynx bears on its outer border five membranelles (Fig. 1) which converge toward its center.

The macronucleus extends along the left side (Fig. 1). It may conveniently be described as composed of two lobes which are separated by the micronucleus. The posterior lobe projects well into the uncinus. In relatively few fixed specimens the anterior lobe appears divided into two fairly equal parts and the posterior lobe into three parts.

The ectoplasm and endoplasm are not markedly differentiated. A thin, fairly clear, non-granular cortex is not uncommonly evident, although in some living individuals the food vacuoles and *Exkretkörner* invade even this clearer boundary. The pellicle is also relatively obscure. It must possess considerable rigidity, however, since the form of the body remains constant.

There is no contractile vacuole in *U. uncinata*, although two or more fluid vacuoles may appear promiscuously in some individuals. Calkins (1911) describes a contractile vacuole for the European species *U. transfuga*, but Young (1922) notes its absence in each of the three species of *Uronychia* occurring at Woods Hole.

The behavior of *U. uncinata* is similar to that described for other species (Calkins, [1911], Young, [1922]). During undisturbed moments, the organism glides leisurely forward over the substrate or in and out among débris by means of its exceedingly active anterior

membranelles and the four ventral cirri. All nine giant cirri remain altogether immotile during these gliding, forward movements. Upon meeting an obstacle, the avoiding reaction, characteristic of the ciliates, is effected in a jerky backward movement for a distance about equal to the animal's length, partly by a reversal of the effective stroke of the anterior membranelles, but chiefly by means of the giant cirri. When disturbed, it dashes, sometimes at a terrific speed, always backward through the water, not infrequently disappearing beyond the low-power field of the microscope. This surprising speed and distance is undoubtedly accomplished mainly by the vigorous strokes of the giant cirri.

SPECIFIC CHARACTERS OF URONYCHIA UNCINATA

The following characters distinguish *U. uncinata* from the three other described species of that genus—*U. transfuga* O. F. Müller, *U. setigera* Calkins, and *U. binucleata* Young—sufficiently, it would seem, to warrant giving this form the rank of a new, fourth species.

Dimensions: *U. uncinata*, 140–50 μ long, 85–90 μ wide, 25–30 μ thick; *U. transfuga*, 130–70 μ long, 100–20 μ wide, 25–40 μ thick (Young, 1922); *U. setigera*, 40–50 μ long, 25–35 μ wide, 10–15 μ thick (Young, 1922); *U. binucleata*, 60–80 μ long, 50–55 μ wide, 20–35 μ thick (Young, 1922).

The prominent uncinus, described in foregoing paragraphs for *U. uncinata*, would appear to be its most distinctive earmark. In Wallengren's (1901) figure of *U. transfuga*, which, as Young (1922) points out, differs considerably from the *U. transfuga* figured by Calkins (1911), the posterior end of that species shows *within* its rounded contour an outline of structure which is suggestive of the uncinus of *U. uncinata*. It will be seen in the figure (Fig. 1) of the latter species that the uncinus projects well beyond the general contour of the body and that its exceedingly acute point is deflected out. One may readily demonstrate this by means of the microdissection needle.

U. uncinata has a prominent oral lip, more so than that figured by Wallengren (1901) for *U. transfuga*. Young (1922) notes the absence of a prominent oral lip in each of the three species at Woods Hole.

The number of giant cirri in *U. uncinata* is invariably nine, arranged in a dorsal group of three and a ventral group of four on the posterior right, and a ventral group of two on the posterior left. The number in each of these groups is respectively the same as that described and figured in Young's (1922) *U. binucleata*, but their relative size, shape, and position are different.

There are four "ventral" cirri in *U. uncinata*, which differ in number and size from those described for any of the other three species of *Uronychia*.

The cytostome is longer and somewhat more spacious in *U. uncinata*. Its undulating membranes are also relatively larger. Cirruslike structures have been observed in the cytostome of *U. uncinata* similar to those figured for *U. setigera* by Young (1922), but such structures appear here rather to be variable folds in the undulating membranes.

The ten membranelles in *U. uncinata* differ to some extent in number and arrangement from those described for the other species.

The macronucleus in *U. uncinata* typically consists of an anterior and a posterior lobe between which lies the single micronucleus. The posterior lobe extends well into the uncinus. This nuclear complex is apparently another fairly specific character.

BINARY FISSION

Wallengren's thoroughgoing researches (1900, 1901), which confirmed Stein's important discovery (1859), showed that the process of binary fission in various hypotrichous ciliates involves the resorption of all motor organelles and the outgrowth of an entirely new set in each of the resulting daughter individuals. These contributions have served as the basis for further careful studies on other ciliated Protozoa such that this reorganization process during fission is now commonly regarded as characteristic for all the ciliates. Calkins (1926) compiles evidence which makes it probable that similar profound changes recur during binary fission in all the main groups of the Protozoa.

One of the hypotrichs thus described by Wallengren (1901) was *Uronychia transfuga*, wherein all cirri, membranelles, and undulating membranes share the same fate during its division. Young (1922)

records similar resorption changes for each of the three species of *Uronychia* indigenous at Woods Hole.

The present investigation has shown that *U. uncinata* undergoes, during its fission, the same sort of protoplasmic reorganization as that described for other species of this genus.

In laboratory cultures maintained for three months at the Hopkins Marine Station, with temperature at about 16° C., *U. uncinata* divided approximately every 34 hours. This period represents an average of the division rate of ten individuals chosen at random.

One of the first readily visible signs of oncoming fission is, as Calkins (1911) points out for *U. transfuga*, the appearance of "buds" which mark the beginning outgrowth of new cirri. Previous to this, however, in *U. uncinata*, one may detect a fimbriation of the giant cirri which I have observed to be progressive, especially noticeable in the group of four. The start of this progressive change is evident even 10–12 hours previous to the appearance of the buds. It continues gradually until the beginning of the constriction of the body in the plane of fission when all of the giant cirri may have become broken up more or less completely into their component cilia. This process of progressive fimbriation is interpreted here as a process of dedifferentiation which culminates in the complete resorption of all motor organelles. Wallengren (1901, p. 23) briefly mentions a comparable procedure in a dividing *Euplotes harpa: Im Anfang und während dieses Resorptionprocesses habe ich oft beobachtet, dass in den Cirren ziemlich deutlich eine fibrilläre Structur hervortritt und dass sie sehr leicht in ihre cilienähnliche Constituenten aufgelöst wurden.*

Another indication of approaching fission was first noted by Stein (1859) and later by Wallengren (1901) in *Stylonychia*. Previous to the appearance of organelle buds, the ectoplasm thereabout loses its granular opaqueness and the pellicle locally "dissolves," thereby giving rise to fairly clear areas in which the buds form and emerge. Such areas are less evident in dividing *U. uncinata*, although I have been able to identify them in a few cases. If sufficiently visible, they should serve as exceedingly useful markings in experimentation.

The relative positions of origin of the locomotor organelles have been admirably described and figured for dividing *U. transfuga* by

Wallengren (1901). These positions, together with the time and manner of growth of the organelles in that species, apparently correspond so nearly with what I have thus far observed in *U. uncinata* that any detailed account of such similar changes is rendered unnecessary.

The remarkable anterior origin of the new giant cirri (Fig. 2) and their subsequent posterior migration happen alike in both species. Similarly, the appearance of the new membranelles and new undulating membranes, which also gradually move, the former anteriorly (Fig. 2) and the latter medianly, into their allotted places, gives due occasion for wonderment in our present want of knowledge of the factors there involved.

One interesting fact which, so far as I can find, has not been described for other *Uronychias* or for any other ciliate, is the single origin of two of the outgrowing giant cirri. Figure 2 illustrates this for the group of two on the left. At first, a single bud makes its appearance median and anterior to the inner one of the old giant pair. A half-hour or so later, this bud bifurcates and so, by continued splitting along with growth, gives rise to the two separate cirri of about equal size, characteristic of the adult stage. A similar behavior has been once observed in the origin and development of the two more median cirri of the group of four.

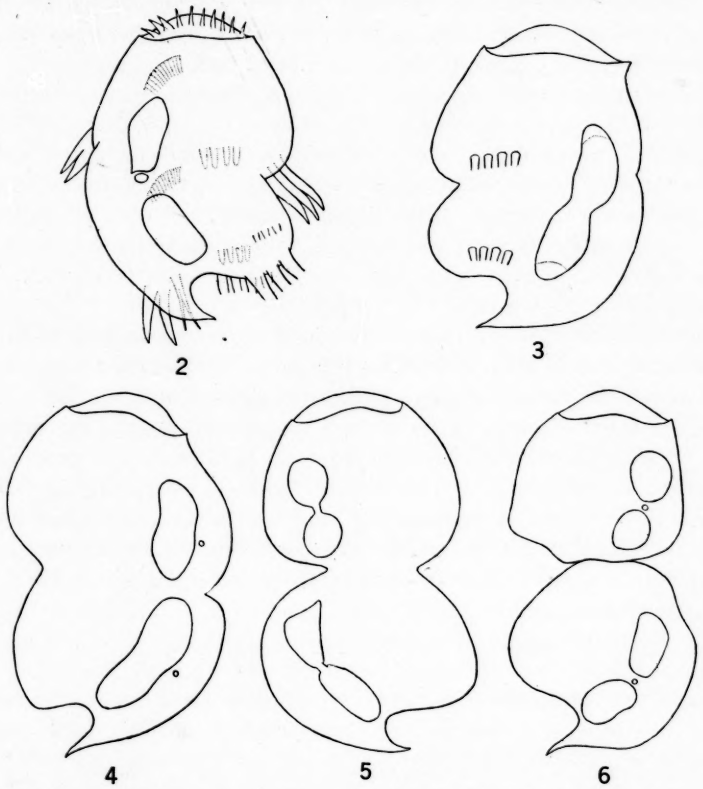
Figures 3–6 show stages of the nuclear changes during fission.

REGENERATION

After making a careful study of the structure and binary fission of *U. uncinata*, microdissection experiments were performed and recorded on more than 200 individuals. Of this number, however, only 68 operated cases were examined step by step throughout the period of their visible regeneration. For these 68 individuals, controls were run, full notes were recorded, and various sketches were made from time to time. The results serve as the basis for the following descriptions and conclusions.

When it had been found for this species that regeneration involved a reorganization of the entire organism, a considerable number of preliminary experiments were undertaken in order to ascertain

the usual time required for the visible onset of the process. It became evident, however, that this latent interval—i. e., the time inter-



FIGS. 2–6.—Binary fission. Fig. 2, camera drawing of a stained preparation showing the relative positions of the various groups of motor organelles, including the bifurcated bud of the forthcoming giant pair; ×1,050. Figs. 3–6, camera drawings of fixed and stained material showing stages in the nuclear complex during binary fission; ×1,050.

val between the operation and the first signs of beginning reorganization—varied as much as 14–22 hours. As will be seen in later paragraphs, there is some evidence to indicate that variation in this

latent interval may be directly referable to: (1) the age of the individual, dating from the completion of fission; (2) whether the regenerating piece were the anterior or the posterior part of the animal; (3) whether the operated animal were an anterior or a posterior daughter; and (4) the degree of the injury. Older individuals begin regenerating sooner; regeneration of the anterior piece begins earlier than that of the posterior piece; the anterior daughter may start regenerating somewhat sooner than does the posterior daughter; and, as noted by Dembowska (1925) for *Stylonychia*, the latent interval tends to vary inversely as the degree of injury.

To establish beyond question each of the foregoing points for *U. uncinata* will require more data than can at present be offered. It may be stated, however, that none of the results thus far give evidence to the contrary, except as regards the latent interval for the anterior daughter as compared with that for the posterior daughter, whereof one rather carefully studied case gave opposite results.

Without exception in more than 200 experiments, the regeneration of *U. uncinata* involved the same sort of protoplasmic reorganization as that which regularly occurs during its binary fission. This was indicated in the regenerating piece by the complete resorption of the old locomotor organelles—cirri, membranelles, and undulating membranes—and the outgrowth of an entirely new set in the reforming individual.

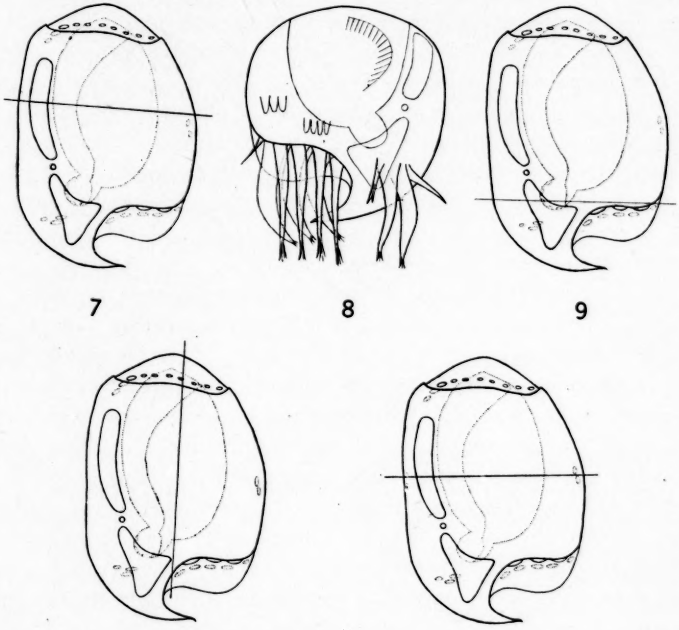
These operations on *U. uncinata* which yielded evidence in support of the above conclusions varied widely both in kind and in degree. For convenience this evidence will now be considered under the following sub-headings: (1) "Transections," whereby the animal either before or during fission was cut into two or more pieces through a plane passing parallel, perpendicular, or diagonal to the longitudinal axis; (2) "Incisions," including minor or major cuts into but not through the animal; and (3) "Excisions," resulting in the removal of part or all of a locomotor organ or of a small fragment of the body.

The relatively few experiments briefly given below have been selected as typical cases from the list of the 68 operated individuals which were successfully studied, some under high magnification (oil immersion), throughout the period of their reorganization.

TRANSECTIONS

EXPERIMENT 8

The animal was transected, 6 hours after the completion of previous fission, through a transverse plane just anterior to the two



FIGS. 7–11.—Regeneration. Fig. 7, plane of transection just posterior to the two small ventral cirri, as in Exp. 8. Fig. 8, a regenerating posterior piece; the giant cirri are undergoing progressive fimbriation along with the outgrowth of a wholly new set of motor organelles; ×1.050. Fig. 9, transection made immediately anterior to the giant cirri, so that the anterior piece contained the micronucleus (Exp. 12). Fig. 10, transection as in Exp. 23 parallel to the longitudinal axis; the right piece, having no nuclear material, did not regenerate. Fig. 11, the plane of transection through both anterior and posterior daughters in Exps. 41*a* and 41*b*.

small ventral cirri (Fig. 7). The anterior piece which contained the membranelles, a portion of the undulating membranes, and part of the macronucleus, but no micronucleus, disintegrated after 28 hours

with no visible signs of regeneration. (Taylor and Farber, 1924). The posterior piece, having the micronucleus, a portion of the macronucleus, parts of the undulating membranes, the nine giant cirri and the four small ventral cirri, completely regenerated after 22 hours, with the resorption of its locomotor organelles and the outgrowth of an entirely new set in the reformed individual. Seventeen and one-half hours after the operation minute buds appeared which, as in binary fission, mark the beginning of new cirri. Their origin was considerably anterior to the bases of the old cirri. The latter, in turn, had already begun a progressive fimbriation. Along with growth, the newly forming cirri very gradually migrated toward the bases of the old cirri. Meantime, a fairly distinct furrow appeared anteriorly on the left in which, some few minutes later, occasional movements of exceedingly minute, closely set buds attracted one's eye. The groove lengthened and more buds readily appeared therein. Back of the more posterior end of the groove a rather narrow, S-shaped, more transparent streak was seen, which slowly became wider and longer. This marks the beginning of the new cytostome, although, as Maupas (1886) observed for the reformation of the new cytostome in the conjugants of *Euplotes patella*, it at first very probably lies beneath the pellicle on the ventral side. So far as I could make out, it appears that with the resorption of the pellicle which bounds the floor of this cytostomal cavity, a thickening ridge comes into view on either side of the cavity. Out of each ridge emerge the cilia which soon fuse to form the right and the left undulating membranes. The membranelles migrate gradually to the anterior periphery, where they project forward. Ventral to their bases the pellicle and peripheral protoplasm grow out to form the characteristic oral plate.

Figure 8 shows a stage in the regeneration of a posterior piece. The giant cirri are undergoing progressive fimbriation, and new motor organelles are appearing.

These more conspicuous features, which are the visible manifestations of a profound protoplasmic reorganization of the regenerating organism, culminate with the complete resorption of the old motor organelles and the full growth of the new set.

Attention should be called to the single bud which, in regenera-

tion precisely as in binary fission, upon bifurcating gives rise to the left pair of giant cirri (cf. Figs. 2 and 8).

EXPERIMENT 11

Nineteen hours after fission the animal was transected through a transverse plane approximately the same as that in Experiment 8. Fourteen hours later the anterior piece, although without a micronucleus, showed the beginning outgrowth of the giant cirri and of the new series of membranelles. Regeneration was complete after about 4 hours. The posterior piece did not give rise to the buds of the new giant cirri until 15½ hours after the transection.

These results differ from those in Experiment 8 in that here both anterior and posterior pieces regenerated—noting that the organism of Experiment 11 was 13 hours older than that of Experiment 8 (Calkins, 1911). It will be observed also that the anterior piece began regeneration about 1½ hours sooner than did the posterior piece, although the duration of the process was nearly the same for each piece.

EXPERIMENT 12

Age of animal, 12 hours. The plane of section was just anterior to the bases of the giant cirri (Fig. 9), such that the micronucleus was contained in the anterior piece; whereas in each of the two cases recorded above, the posterior piece had the micronucleus. In this experiment the posterior piece died after some 20 hours, without any indication of regeneration. In the anterior piece, 17 hours after the operation, buds of giant cirri and of membranelles were clearly evident. The completion of regeneration required nearly 5 hours.

EXPERIMENT 23

Sixteen hours after fission, the transection was made parallel to the long axis producing two fairly equal pieces (Fig. 10). The right piece, which contained none of either nucleus, died after about 27 hours. The left piece, having all of both nuclei, the left pair of giant cirri, most of the left undulating membrane, and four or five membranelles, showed the first appearance of buds about 15 hours after the operation. The process was completed in about 5½ hours.

Regeneration did not occur in the non-nucleated piece; but

whether or not regeneration depends on the presence of the micronucleus, and at least part of the macronucleus, remains to be determined not only for *U. uncinata* but for other ciliates also.

EXPERIMENTS 41*a* AND 41*b*

Nine hours after fission the anterior and posterior daughters were each cut transversely through approximately the same plane, so as to divide each daughter into fairly equal anterior and posterior pieces (Fig. 11). The posterior piece of each contained the micronucleus and part of the macronucleus. Each posterior piece regenerated; but that of the anterior daughter began its regeneration fully 2 hours earlier than did the equivalent piece of the posterior daughter.

The anterior piece of each died (Calkins, 1911; Young, 1922), but no record was made of the time.

Two such cases are recorded with results about as stated above. Yet one experiment, No. 43, gave opposite results. The posterior equivalents regenerated, but the regeneration of the posterior daughter began about three quarters of an hour before that of the anterior daughter.

INCISIONS

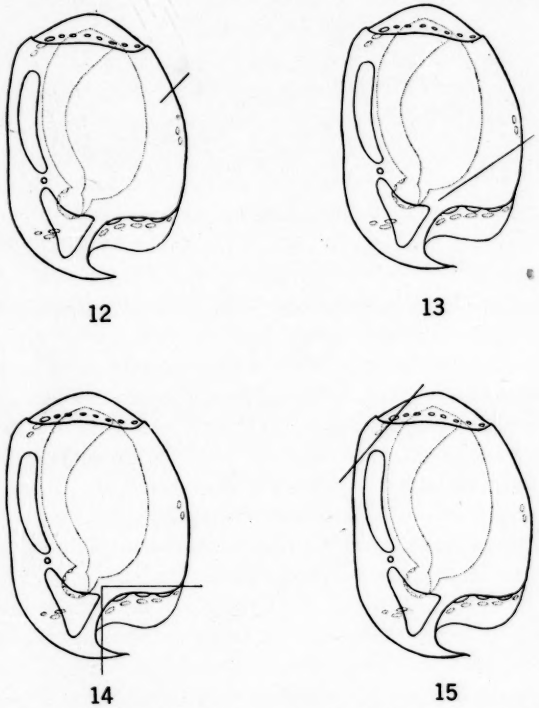
EXPERIMENT 9

A shallow incision was made in the right side of an animal of unknown age (Fig. 12). No cirri, nuclei, or undulating membranes were affected in the operation. The wound healed completely in about $1\frac{1}{4}$ hours, although the behavior of the animal was not entirely normal for at least 3 hours after the wound had apparently fully healed. No reorganization was induced during 26 hours after the operation.

EXPERIMENT 17

A much deeper incision than that in Experiment 9 was made also in the right side of another individual, somewhat more posterior and directed diagonally backward (Fig. 13). Cirri, undulating membranes, and nuclei were not affected in the operation. Beginning of outgrowth of cirrus buds was observed 15 hours after the operation. Regeneration was practically completed 4 hours later. Comparing

these results with those of Experiment 9, it is evident that regeneration is here to be correlated with the degree of injury resulting from the incision wherein the motor organelles were not directly affected.



FIGS. 12–15.—Regeneration. Fig. 12, a shallow incision (Exp. 9) which did not injure any of the motor organelles; the wound "healed," but reorganization did not ensue. Fig. 13, a deep incision (Exp. 17) which did not directly injure any of the motor organelles, followed by a reorganization of the entire organism. Fig. 14. Excision, as in Exp. 44, of the group of three and the group of four giant cirri; reorganization occurred. Fig. 15, excision (Exp. 38) of left anterior corner including three membranelles, followed by reorganization of the entire organism.

Dembowska (1925) concludes that regeneration in *Stylonychia* is dependent on a "disturbance of the coördination of locomotor organelles." Any such producible disturbance in *U. uncinata* apparently can be effected without direct injury to the motor organelles, al-

though it might result from injury to neuromotor fibers (Taylor, 1920), which I have thus far failed here to observe, and Young (1922) states that none were evident in any of the three species at Woods Hole.

EXCISIONS

EXPERIMENT 44

All giant cirri, except the left pair, were removed from an individual 14–15 hours old (Fig. 14). Regeneration ensued about 16 hours after the excision. The time of its duration was not observed.

EXPERIMENT 38

The upper left corner of the organism including three membranelles was removed (Fig. 15), resulting in the onset of reorganization 18½ hours after the operation. Regeneration was completed in about 5 hours.

EXPERIMENT 57

The outermost giant cirrus of the group of three was excised, including a bit of the protoplasm at its base. Fifteen hours later, regenerating cirrus buds had made their appearance. Duration of the process was 6–7 hours.

EXPERIMENT 62

In another individual, the base of the same giant cirrus as that excised in Experiment 57 was injured considerably by means of the needle tip, so that the cirrus then projected outward at an angle of perhaps 15° to that of its normal position. Reorganization ensued after 18½ hours.

EXPERIMENT 66

The same giant cirrus in another individual was carefully excised so that approximately one-half of it remained intact, apparently without further injury. This stub persisted and was, part of the time at least, normally active until the animal underwent normal fission about 38 hours later. The regeneration process was not induced. Young (1922) also noted that such a stub did not regenerate. He does not mention, however, having observed the reorganization process, described above, to occur in regenerating *Uronychia* of the species upon which he experimented.

DISCUSSION

It has here been experimentally demonstrated that regeneration in *Uronychia uncinata* involves a profound protoplasmic reorganization which is altogether comparable with the well-known changes that take place in the Ciliata during binary fission. All external motor organelles—cirri, undulating membranes, and membranelles —are entirely resorbed (dedifferentiated) in the regenerating piece, and a new set of these complex structures is at the same time redifferentiated in the reforming organism.

These significant results followed operations which varied both in kind and in degree. Whether regeneration involved: (1) the replacement of a part of the organism which had been transected through a plane parallel, transverse, or diagonal to the long axis, or (2) the reformation of a cirrus excised, or even severely injured, at its base, or (3) the healing of deep (but not of slight) incisions made at various points, in all carefully studied cases (totaling 68) this remarkable reorganization process ensued—the dedifferentiation of the old organelles and redifferentiation of a wholly new set.

It was pointed out that similar results were obtained in *Euplotes patella* (Taylor, 1923) and in Stylonychia (Dembowska, 1925). Accordingly, it seems reasonable to suspect that these deep-seated changes will be found to accompany regeneration in many other, probably all, ciliated Protozoa. It is obvious that such changes can be detected more readily in hypotrichous Protozoa whose cilia are commonly grouped in conspicuous cirri, membranelles, and undulating membranes. There are, however, many other ciliates (e. g., *Lembadion*, *Condylostoma*, *Pleuronema*, *et al.*) which possess prominent undulating membranes or membranelles that ought to afford fairly ready detection and accurate analysis of this exceedingly interesting protoplasmic change if it takes place there also during regeneration.

There remains, too, the study of internal organelles, such as fibrils and nuclei, which may very possibly undergo changes during regeneration similar to those described above for external motor organs.

Should it be demonstrated that this procedure of protoplasmic

reorganization during regeneration is characteristic for all the differentiated structures, internal and external, of the ciliates, then certain relations that concern its fundamental significance would seem to be clear. Whatever basic factors underlie the universal and extraordinarily complex phenomenon of organization in living matter, we can observe the grosser nature of that organization, especially in the Protozoa. We can see there very diverse, highly differentiated organs performing specific, yet beautifully co-ordinated functions. Food vacuoles, fluid and contractile vacuoles, nuclei, fibrils, locomotor organs—these and other grosser structures we know to be made up of more or less markedly diverse, molecular components, yet all operate in utmost rhythm and harmony as if in magical response to some directive, subsisting entity.

Some such picture of a living, active organism, while altogether crude and inadequate, helps to elucidate one's meaning of organization in living matter—an organization which may be expressed as the differentiation and integration of diverse structures performing specific and co-ordinated functions. Accordingly, the organism is the product, rather than the sum, of its parts.

Bearing this concept of the organism in mind, it is less surprising that injury to, or excision of, one or more of its highly differentiated parts should produce even profound changes in all other parts with which it is integrated. How far-reaching these changes are, is evident from the experimental results herein described.

Precisely similar changes, it will be recalled, accompany the process of binary fission. Also, it has been shown by Maupas (1889) and others that entirely comparable changes occur in conjugating *Euplotes patella* and in certain other ciliates. Furthermore, the early work of Stein (1854) on *Epiclintes*, and subsequent researches of other authors, have made it evident that similar resorption phenomena take place during the encystment of various ciliates. Recent careful studies of induced encystment and excystment in *Euplotes* (Garnjobst, in press) indicate that dedifferentiation and redifferentiation of all motor organelles there also result.

In the ciliates thus far studied, these two basic yet reverse processes—dedifferentiation and redifferentiation—ensue during binary fission, regeneration, and conjugation apparently at much the same

time but in different regions of the protoplasmic mass. These outward manifestations, however, may be regarded as the culmination of profound changes throughout the protoplasmic mass that have been under way relatively long before these visible signs become evident.

Parallel instances of a common process which may be assumed to recur normally in phenomena that otherwise appear to be so diverse as do encystment, conjugation, binary fission, and regeneration, suggest that such a process is of fundamental significance. Moreover, if that process involves all of the differentiated structures throughout the protoplasm, we may well afford to look for comparable phenomena in other Protozoa, even in cells of the Metazoa. It is altogether plausible to regard the cyclic protoplasmic changes which take place during mitotic division in metazoan cells as phases of reorganization that are quite comparable with the dedifferentiation and redifferentiation processes herein noted for regenerating, dividing, conjugating, and encysting ciliates. The appearance, disappearance, and reappearance of the mitotic figure, of the nuclear wall, of vacuoles, of chromosomes, of cilia, of ciliary rootlets, of basal granules, and of various other differentiated protoplasmic structures, suggest that these cyclic changes fundamentally share much in common with the protoplasmic reorganization that recurs in these diverse phenomena of various ciliates. If this point of view should prove to be tenable, then we are concerned here with a basic process that may rightly come to rank among the common properties of protoplasm.

The bearings which this cylic process of reorganization in living matter may have upon some general biological problems appear to be more evident when viewed in the light of certain colloidal properties of matter. Physically, the differentiation of a protoplasmic structure, such as a vacuolar "wall" (Taylor, 1923*b*), a nuclear membrane, a chromosome, an axial filament, or a basal granule, may, in its simpler aspects, be regarded as involving a condensation of the molecular components which previously, in their dedifferentiated state, had assumed a more dispersed condition characteristic of the general structure of the protoplasm throughout the reorganizing cell. The researches of Langmuir (1917), Harkins (1917), and others indicate

that such condensations result in a tendency toward a more restricted and definite orientation of the approximated molecular components, especially at surfaces or interfaces, and Hardy (1927) has recently pointed out that this tendency theoretically exists throughout a delimited mass. Dedifferentiation of a protoplasmic structure may then be regarded as physically a dispersion process—the return of the molecular components of the differentiated structure to the more dispersed condition of the reorganizing protoplasm. In the redifferentiation of a cilium, a cirrus, a membranelle, a basal granule, or even a chromosome, it is readily conceivable that the physical "recondensation" process might involve a somewhat new arrangement, or a new combination, of the molecular components, thus bringing about a more or less visibly changed structure, hence a "variation." Variations that may represent a resultant of such a reorganization process are everywhere evident in living things. No two daughter *U. uncinata* are quite identical, either to each other or to the mother organism from which, by fission, they were derived. In a chain of dividing dinoflagellates, such as *Ceratium* (Kofoid, 1909), individual differences are sometimes striking. Slight, fluctuating variations of this sort, assuming that they may take origin from a reorganization process such as noted above, may have their counterpart in other more striking, more permanent variations which, having a similar mode of origin, owe their permanence to more stable combinations of unique components such as genes.

Again, Calkins (1926) has recently re-emphasized Child's (1916) theory of rejuvenation accompanying binary fission and mitotic division. Child maintained that normal accumulations of metabolic products within the cell tend to decrease the metabolic rate of that cell with a resulting senescence, unless counteracted. Child saw in cell division such counteracting processes due to reorganization. "Senescence consists in a decrease in metabolic rate determined by the change in, and the progressive accumulation of, the relatively stable components of the protoplasmic substratum during growth, development and differentiation" (Child, 1916, p. 333).

Calkins, in support of this hypothesis, has pointed out various striking instances of periodic reorganization in all the chief groups of the Protozoa, and concludes (p. 242) that this evidence "may be in-

terpreted as supporting Child's view of dedifferentiation as an offset to the accumulation of products of metabolism which hamper further metabolic activities."

If we should suppose that this dedifferentiation process involves a dispersion of the molecular components of the various differentiated structures throughout the dividing cell, then the ensuing redifferentiation process might very conceivably bring about a new condition in the recombination of formerly dispersed materials and the elimination of those of metabolic origin which tend to "decrease the metabolic rate."

If the elimination of these deleterious accumulations is thus effected by some such recombination process during protoplasmic reorganization, then rejuvenation should ensue whenever and wherever protoplasmic reorganization occurs. Not only should daughters resulting from binary fission be rejuvenated, but so also should all exconjugants, all excysted, and all regenerated individuals. Calkins (1926) offers considerable evidence which indicates that rejuvenation does follow in all such cases.

SUMMARY

1. *Uronychia uncinata*, n. sp., is described.

2. Binary fission in *U. uncinata* is accompanied by protoplasmic reorganization such as commonly occurs in the Ciliata. This includes the resorption of all old motor organelles and the outgrowth of a new set in each of the resulting daughters.

3. Regeneration in *U. uncinata* involves protoplasmic reorganization which is entirely comparable with that taking place during its binary fission. All old motor organelles are resorbed, and a new set appears in the regenerating piece.

4. Reorganization thus followed operations which included: (1) transections of the organism through a plane parallel, perpendicular, or diagonal to the long axis; (2) deep incisions into the animal, which did or did not affect its motor organelles; (3) excisions of all of one or more motor organs; and (4) the injury of a single giant cirrus at its base.

5. Reorganization did not ensue upon making slight incisions or when only a portion of a giant cirrus was excised.

6. A condensation-dispersion theory of protoplasmic reorganization is proposed, correlated with redifferentiation and dedifferentiation during binary fission, regeneration, conjugation, encystment, and other cyclic protoplasmic changes. The bearing of this theory upon certain biological problems, such as those of variation and rejuvenation, is discussed.

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STUDIES OF CELL DIVISION. II. THE PERIOD OF MAXIMUM SUSCEPTIBILITY TO DILUTE SEA-WATER IN THE CLEAVAGE CYCLE OF THE FERTILIZED EGG OF ARBACIA

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THE problem of mitotic cell division, despite years of intensive study which has given rise to much theorizing, is today far from solution. With respect to that part of the problem which relates to the division of the cell body, we find two main groups of postulates: the first explains division in terms of the astral configuration (when this is present); the second, in terms of the behavior of the cell surface.

That the leading rôle in cell division is played by the astral system (not necessarily present in mitotic cell division—e.g., most plant cells and the maturation divisions in eggs of *Ascaris*, *Ciona*, etc.) is supported by the studies of Chambers[1] and more recently by

[1] Unfortunately, as Heilbrunn has pointed out, Chambers' 1917 and 1919 accounts of viscosity changes during mitosis are not in strict agreement. Heilbrunn has justly, it seems to me, adversely criticized the microdissection method for measuring changes in viscosity. Easily enough can one determine the difference in viscosity between a volume of water and one, say, of molasses. But how one can "feel," as Chambers is so fond of saying, changes in the living substance with the fine needle mechanically fixed is beyond my comprehension. And Chambers himself is uncertain, as his papers show. The microdissection method, Heilbrunn has pointed out, is far too subjective adequately to measure physical changes in the living cell. In addition, eggs in a hanging drop pushed up into the angle between the water film and the glass surface suffer deformation and are apt to be abnormal. If, now, such eggs are studied under the high power illuminated with light from an arc lamp without cutting out the heat, they are further injured. Added injury through puncturing them with a needle would thus be unnecessary. But these three methods of abuse combined would certainly guarantee results. Actually, Chambers' microdissection studies have added little to our knowledge of the physical state of protoplasm. At most he has used this method and anew presented old descriptions; or, he has repeated old experiments (like those of Yatsu, etc.) made more easily with an iridectomy knife. See too the studies of Hyman, Tharaldsen, and Fry.

those of Gray. Says Chambers (1919): "The comparative firm consistency that the egg now attains is due to two masses, the two asters." And later: "The two spheres of solidification grow at the expense of all but possibly a *small peripheral part of the fluid egg substance*" (italics mine). The division of the egg into two blastomeres, he concludes, takes place "when *all the cytoplasm* is incorporated in these two masses" (italics mine). According to Gray (1924):

> Since the asters are in fact areas capable of resisting pressure, and are more viscous than the peripheral endoplasm, cleavage of the cell must tend to be visible as soon as the diameter of each aster is half that of the whole cell and the pressure exerted by them overcomes that of the ectoplasm. The extent to which the egg will be deformed by elongation will depend upon the degree to which the asters resist deformation by pressure, and the degree to which the ectoplasm [hyaline plasma layer] resists stretching.

Both authors consider elongation of the egg due to the growing astral systems.

On the other hand, there are strong proponents of the theory that surface changes explain division of the cell body. Thus, changes in surface tension, which constitute a veritable *deus ex machina* piously invoked by many biologists to explain various phenomena in living cells, make up one such theory.

The surface-tension theory of cell division has gained adherents largely from experiments on oil drops (McClendon, Robertson, Spek) and from R. S. Lillie's results on the effect of hypotonic sea-water on the living cell during the division cycle. In passing, we may note that these oil-drop experiments lead to opposite conclusions: McClendon interprets them to mean that the living cell divides because of reduction of surface tension over the spindle poles; while Robertson makes them prove a reduction of surface tension at the equator, in the site of the future cleavage plane. Spek's oil-drop experiments confirm McClendon; while Lillie's work (1916) on the egg of *Arbacia* leads him to the conclusion that there is an increased surface tension over the spindle poles. (See also, Lillie, 1903.)

Thirty years ago von Erlanger made beautiful observations on the streaming movements in the living cell during division. Conklin has shown that in the egg of *Crepidula* peripheral yolk spheres move

from over the spindle poles toward the equator and thence along the plane of the future cell wall. The movement of pigment granules along the cleavage furrow had been previously described by Nussbaum, Gardiner, Loeb, and others. More recently, Spek's important studies (1918) have confirmed and extended this older work on protoplasmic currents in the living egg. Spek's observations, because analogous to those on oil drops, are held as giving strong support to the surface-tension theory of cell division.

My published data on the egg of *Echinarachnius* (Just, 1922) showed that the centrospheres are largest and the astral rays most widely extended in the late anaphase and the early telophase while the egg is still spherical—that is, before the period of elongation. They thus furnish evidence in contrast to the findings of Chambers and of Gray. However, my observations on this point are in accord with most descriptions of mitosis in echinid ova—for example, in those of *Toxopneustes, Cidaris, Strongylocentrotus*, etc.: in these ova also the centrospheres and asters are most pronounced during the anaphase and early telophase while the egg is still spherical; elongation follows rapidly, and after this in turn the egg divides. One illustration will suffice.

Perhaps the most popular account of mitosis in the sea-urchin egg is that by Wilson (1895). Wilson's Figure 17 and Phototype 34 show the egg still spherical in the early telophase of first cleavage, with chromosomes as vesicles, centrospheres at their maximum size, and astral rays widely extended. Figure 18 and Phototype 35 show the egg elongated in process for cleavage with the chromosome vesicles fusing within the shrunken centrospheres and the astral rays reduced. Figure 19 and Phototype 36 show the divided egg with fully formed daughter nuclei. Thus, too, the egg of *Echinarachnius*.

It may be that students of the mechanics of mitosis in their attempts to explain this phenomenon err in basing their explanations on textbook diagrams rather than on carefully controlled observations on normal living and properly fixed cells. Such diagrams are apt to be erroneous. It is a curious fact that Wilson himself is guilty of a serious error in figuring (page 120 of *The Cell in Development and Heredity* [1924]) a diagram purporting to be the closing stage of

mitosis in the sea-urchin egg. I refer to Figure 47*Hb* which bears no likeness to his own descriptions cited above.[1]

In what follows we shall see that the egg of *Arbacia* resembles that of *Echinarachnius* and of other echinids with respect to the time of maximum size of the astral configurations.

It is alleged[2] that my observations on the effect of dilute sea-water on the egg of *Echinarachnius* during the cleavage cycle support the theory of increased surface tension at the equator of the cell as the "cause" of cleavage of the cell body. This allegation rests on no such statement of mine. I did find that this egg during its division cycle exhibits maximum susceptibility to hypotony by bursting over the poles. I stated that I considered this due to a drop in the tensile strength of the hyaline-plasma layer in these areas: the egg becomes denuded of hyaline-plasma layer. In his study on the rhythmical susceptibility of *Arbacia* eggs to dilute sea-water, Lillie did not observe the point at which the egg ruptures, as is sometimes stated. And yet, were the cytolysis of the egg during the period of maximum susceptibility localized, we should expect it to take place in the region of lowered surface tension—according to Lillie, at the equator.

Again, my observations on eggs of *Echinarachnius* revealed two periods of susceptibility during the cleavage cycle. This is likewise at variance with Lillie's work on eggs of *Arbacia*. Finally, according to this worker, eggs of *Arbacia* are most susceptible at the time of cleavage. This lack of agreement could not be due to method, for I used more dilute sea-water than Lillie used; but it might be due to differences in the eggs. Since I make a point of the fact that the egg of *Echinarachnius* is most susceptible while a sphere and before the period of "mitotic elongation"—i.e., before the egg changes from sphere to ellipsoid—and since Lillie finds that the egg of *Arbacia* is most susceptible as it changes from ellipsoid to two spheres (the two blastomeres), it seemed worth while to study the effect of dilute sea-water on the egg of *Arbacia* during the cleavage cycle.

[1] Compare this diagram with Figures 58*C* and *D* (page 135, of *The Cell*), mitotic stages in the still spherical *Toxopneustes* egg.

[2] Cf. Sharp, *Introduction to Cytology* (1926), p. 209.

EXPERIMENTAL

At the Marine Biological Laboratory, Woods Hole, Massachusetts, I have had the opportunity of making numerous observations on the rate at which inseminated eggs of *Arbacia* disintegrate while in dilute sea-water. These observations indicate that this egg during its cleavage cycle responds to the action of dilute sea-water in a way closely similar to that of the egg of *Echinarachnius*, as this account will show.

The egg of *Echinarachnius* exhibits during its cleavage cycle two periods of susceptibility to dilute sea-water: the first comes on after the appearance of a streak in the region of the zygote nucleus; the second, at the time of the late anaphase or the early telophase while the egg is still spherical, the centrospheres at their greatest size, and the astral rays best defined. The hyaline-plasma layer now thins over the spindle poles and forms a thick girdle around the equator, the site of the future cleavage plane; the egg elongates and is again resistant to hypotony. Cleavage follows. During this second period of susceptibility the egg bursts in dilute sea-water over the area of the spindle poles.

Uninseminated eggs of *Arbacia* exposed to dilute sea-water take up water and swell; if the dilution be sufficiently great, they cytolyze (Lillie, 1916). The time to cytolysis depends upon the degree of dilution. Immediately after insemination, for a period of about two minutes, the egg responds in much the same way. Beginning two minutes after insemination, there now ensues a period of resistance, as shown by Lillie (1916), which drops sharply at the time of cleavage.

One may measure the susceptibility by exposing eggs at intervals after insemination to sea-water of graded dilutions with tap or distilled water, noting the percentage of cytolysis after a given lapse of time (cf. Lillie, 1916, p. 377). Another method used by this worker for demonstrating the period of susceptibility is that of exposing the eggs to the dilute sea-water for a given length of time and on their return to sea-water noting the percentage of development. I have used both methods with concordant results. For sharp, clear-cut results, however, admitting of no possibility that the eggs may for a time after coming into the dilution so withstand the hypotony that

they continue to develop, I find it best to expose them to extreme dilutions at 1- or 2-minute intervals after insemination, watching under the microscope the course of cytolysis and recording in seconds the time to disintegration. This method makes it possible to mark definitely the moment of maximum susceptibility. In this paper only those experiments with the most dilute sea-water are reported.

I should point out that it is most desirable to use eggs in the best possible conditions, thus insuring an even cleavage *tempo*. Therefore, I always tested out the fertilization capacity of the eggs before making an experiment: if membrane separation was uniform with respect to rate and quality, I used the eggs. *Such eggs cleave at a remarkably uniform rate.* Inseminations were always made with a thin, freshly prepared sperm suspension added to eggs in 250 cc. of sea-water.

I cite now one experiment from a group of twelve, in all of which cleavage took place in 53 minutes after insemination. In a number of experiments the eggs cleaved around 45 minutes after insemination; in such cases it is more difficult exactly to fix the periods of susceptibility. In the experiment cited, as in every one made, the eggs used were from a single female.

The experiment cited shows that the susceptibility of the egg of *Arbacia* is not so sharply revealed as that of *Echinarachnius*. Nevertheless, there is a drop in resistance to the action of distilled water during the "pause" which is doubtless correlated with the behavior of the cortex at this time. Succeeding this phase is the "dumb-bell" stage, i.e., the cleavage spindle fully formed. The egg is now resistant.

The period of resistance lasts until just before the elongation which precedes cleavage. The egg, still spherical, the cortex thinning out over the spindle poles to heap up at the equator, enters the period of maximum susceptibility to distilled water. At this time the chromosomes, as anaphase rods or as telophase vesicles, lie at the inner border of the centrospheres; the centrospheres are at their maximum size; and the astral rays widely extended. With the elongation of the egg, resistance returns and persists through cleavage. After elongation, the hyaline-plasma layer extends inward at the equator along the plane of cleavage with pigment granules lined up

on each side; thus the egg divides. In the second cleavage cycle the egg, before division, exhibits a similar drop in resistance.

In the egg of *Arbacia* as in that of *Echinarachnius* the elongation cannot be due to the thrust of the spindle, because all my preparations of these eggs show the spindle fibers broken across their equator

TABLE I

THE RATE AT WHICH EGGS OF *Arbacia* DISINTEGRATE IN DISTILLED WATER AT INTERVALS AFTER INSEMINATION*

No.	Time, in Minutes, After Insemination	Time, in Seconds, to Complete Disintegration	Comment
1.....	3	240	
2.....	6	180	
3.....	10	60	
4.....	15	60	
5.....	17	60	
6.....	18.5	65	
7.....	21	80	
8.....	23.5	75	Streak indicated
9.....	26	70	
10.....	27.5	60	
11.....	29	60	
12.....	31	60	
13.....	33	25	
14.....	35	25	Streak
15.....	37	30	
16.....	39	60	"Dumb-bell stage"
17.....	41	100	
18.....	43	60	
19.....	45	60	
20.....	47	20	Eggs burst over spindle pole
21.....	48	20	
22.....	49	60	Eggs elongate
23.....	51	60	Cleavage furrow indicated
24.....	53	120	Cleavage

* Eggs of *Arbacia* are inseminated, and at intervals after insemination 5 drops of them are mounted under the microscope in 15 cc. of distilled water. The first column gives the number; the second, the time after insemination; the third, the time to cytolysis; and the fourth, the stage of the egg at the time of exposure.

in the anaphase stage (so, too, Wilson on the egg of *Toxopneustes*). Instead, it must be due to streaming movements in the more peripheral endoplasm from over the poles of the spindle, which, meeting at the equator and opposing, are *forced inward* to the spindle area where the fibers have broken down—*by the girdle of hyaline-plasma layer*. This layer of endoplasm, the layer of pigment granules, and the hyaline-plasma layer itself thus separate the blastomeres.

Finally, the experiments also show that the egg of *Arbacia*, like that of *Echinarachnius*, when treated with dilute sea-water *breaks down while it is a sphere immediately before elongation by rupturing over the spindle poles*. I find however, that the locus of rupture is more difficult to fix in this egg. On cool days, when the eggs develop more slowly, the zone of rupture is easier to fix. But at best this is never so clear-cut as in the egg of *Echinarachnius*. A similarly localized rupture in hypotonic sea-water has been described by Conklin for the egg of *Crepidula*. On the basis of this observation Conklin concluded that the so-called "mitotic elongation" could not be due to the thrust of the spindle, since the rupture may take place before the spindle forms.

Summarizing, we find that the egg of *Arbacia* exhibits during its cleavage cycle a most marked drop in resistance to dilute sea-water while still spherical when (1) the spindle fibers are ruptured in their equatorial region, mitosis is at the late anaphase (chromosomes as rods at the inner border of the centrospheres) or at the early telophase (chromosomes as vesicles); (2) the centrospheres and asters are at the height of their development; (3) the hyaline-plasma layer is thinning over the spindle poles to heap up at the equator. Finally, (4) the egg manifests this drop in resistance by breaking over the spindle poles.

DISCUSSION

It is a safe assumption that a theory devised to explain division of the cell body in which the mitotic figure shows astral configuration should be consonant with the observed phenomena in the normal living and properly fixed cell. Physicochemical speculations, however ingenious, inconsistent with such observations, lend little to the solution of the problem. If the observations concerning the relation of the size of the astral systems to stages in mitosis reported above be correct, a few simple deductions follow.

1. As I have pointed out above, the fact that the spindle fibers are ruptured during the anaphase suggests that the elongation of the egg cannot be due to spindle extension. The egg elongates in that stage of the telophase in which the chromosomal vesicles are fusing within, and probably at the expense of, the now greatly shrunken

centrospheres. Theories of cell division fabricated on this so-called spindle thrust are therefore suspect.

2. Cell division in echinid ova, at least, does not take place when the centrospheres and asters are at their maximum size. Wilson's figures also show this very clearly. A theory of cell division based on the astral configuration that ignores the proper sequence of events in mitosis is of little value.

3. Preparatory to the elongation of the egg, the hyaline-plasma layer thins over the poles of the spindle and heaps up at the equator. This thickened girdle of hyaline-plasma layer has been described by others—notably, McClendon. The ctenophore egg also shows increased width of the ectoplasm at the site of the cleavage plane; and it has been suggested that this is but an exaggerated expression of conditions found in other dividing ova. Morphological surface changes are most striking in the egg of *Chaetopterus*—especially during the maturation divisions.

In the egg of *Arbacia*, the thinning of the hyaline-plasma layer over the poles releases restraint from the endoplasm immediately below these areas. The egg does not cleave, therefore, because there are now two solid spheres, the asters (Chambers), the sum of whose diameters is equal to that of the whole egg (Gray). Rather, the egg elongates, then cleaves, because of the movements from circumpolar areas to equator, thence inward across the region of the now wholly disrupted spindle fibers.

With respect to the rhythmical susceptibility during the cleavage cycle revealed by the rate of cytolysis and the locus at which cytolysis takes place while the eggs are in distilled water, two points are clear:

1. My observations on the rate of cytolysis reveal two periods of maximum susceptibility during the cleavage cycle. These parallel very closely surface changes in the sea-urchin egg studied by Vlés. Thus, his curve (Fig. 7, *Tensions de surface évaluées en dynes pendant la division*) approximates a similar curve that might be plotted to show the periods of susceptibility to dilute sea-water manifested by eggs of *Arbacia*. Especially noteworthy is the fact that Vlés's highest value comes just before elongation of the egg. From elongation to and including cleavage this value drops. Thus, our findings are in

more accord with each other than they are with those on viscosity measurements which seem to show a maintenance of high viscosity before and through cleavage (Heilbrunn). Indeed, Vlés concludes that "*les relaxations du système visqueux n'interviennent que d'une façon négligeable.*"

The rate of cytolysis is correlated with structural changes in the hyaline-plasma layer. To me these changes are evidence of a temporary disruption of the hyaline-plasma layer which leaves the egg inadequately protected when exposed to dilute sea-water. Such a temporary breakdown probably means also changes in electrical potential. These changes in the hyaline-plasma layer are rhythmical and are most intense at the time of membrane separation following insemination, at which time there are great increases in oxygen consumption and heat production. If the rate of cytolysis at stages during the division cycle be evidence of changes in permeability, then such changes are due to temporary breakdown of the surface layer.

2. That there is such a temporary breakdown, the locus at which cytolysis of the egg begins while in distilled water seems to indicate: the egg breaks down by rupture over the spindle poles where the hyaline-plasma layer thins out. This is no mere surface-tension change. The hyaline-plasma layer is not simply an inert sheath—it has appreciable width which shows rhythmical variation and an ever changing structure. Changes in surface tension may take place, but these alone cannot explain the division of the cell body.

SUMMARY

1. Eggs of *Arbacia* show a rhythm of susceptibility to hypotony during its cleavage cycle, as measured by the time to cytosis in tap or distilled water and by the percentage of cleavage on return to normal sea-water after exposure.

2. Two periods of low resistance to hypotony are noted; the greater, which is the maximum susceptibility during the cleavage cycle, comes on when the egg is still spherical, chromosomes in late anaphase or early telophase, the hyaline-plasma layer in a highly active state, and astral system at the height of its development. With elongation of the egg resistance returns and remains through cleavage.

3. The egg reveals its maximum susceptibility to hypotony by breaking down over the spindle poles.

4. Theories of cell division must be based on knowledge of the sequence of events during the stages of mitosis. The evidence here presented suggests that peripheral movements and the activity of the hyaline-plasma layer play the leading rôles in division of the cell body.

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SULPHATE RETENTION IN DOGS FOLLOWING BILATERAL ADRENAL EXTIRPATION[1]

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THE results of previous work by the senior writer (1926 and 1927) and his co-workers (Zwemer, 1927; Corey, 1927 Yonkman, 1927; Swingle and Eisenman, 1927) on the functional significance of the suprarenal cortex indicated that probably the cause of death in double-operated cats and dogs is acid intoxication due to retention of acids formed in the course of normal metabolism. The acidosis is apparently not due to the formation of abnormal acids within the organism, but primarily to defective elimination of non-volatile acids. In the earlier work, attention was called to the resemblance between the acid intoxication resulting from adrenal insufficiency and that occurring in the terminal stages of chronic interstitial nephritis, eq. vremia.

In a study of the acid-base equilibrium of adrenalectomized cats, Swingle and Eisenman (1927) determined the serum protein, serum carbon dioxide, serum chloride, inorganic phosphorus, total base, and pH. The acid values of protein, bicarbonate, chloride, and phosphorus were calculated. Total acid was taken to be the sum of all the determined acids, while organic acid was calculated by subtracting total acid from total base and included the sulphate ion. Sulphur determinations were not made because of the large quantity of blood required in addition to that necessary for the other tests. It was not deemed expedient to subject the experimental animals to loss of such considerable amounts of blood.

The results of the acid-base equilibrium tests on normal and operated cats showed quite clearly that the animals showing marked symptoms of adrenal insufficiency were suffering from an uncompensated non-volatile acidosis. According to the data available, the

[1] Part of the expenses of this investigation were defrayed by a grant from the Bache fund of the National Academy of Science.

acidosis was attributed to an increase in phosphoric and undetermined organic acids. However, it was evident that the marked fall in bicarbonate observed in our double-operated animals was not occasioned to any considerable extent by the rise in inorganic phosphorus, which, although marked, never exceeded 2 millimols, whereas the carbon dioxide fell from 6 to 10 millimols. We stated that "increase in organic acid [which in our data included the sulphate ion] is the one change of sufficient magnitude to be responsible for the bicarbonate fall."

Recent experiments performed in this laboratory on dogs by Yonkman (1927) have likewise shown that the rise in inorganic phosphorus is not sufficient to account for the degree of acidosis observed.

Further efforts on our part to determine the nature of the organic acid or acids responsible for the fall in bicarbonate ended in failure; hence, it seemed worth while to test for sulphate retention to see if the sulphate ion could be responsible for the observed changes, since in the original work the sulphate ion was lumped together with organic acid. The idea seemed especially likely to prove fruitful since earlier experiments by Swingle (1927) had shown that the kidney function is abnormal in adrenal insufficiency. The work of Denis (1923) and others had also demonstrated that the sulphate ion is apparently difficult for the kidney to eliminate even under normal conditions and is retained in considerable quantities in certain types of nephritis.

Dogs were chosen for experimental animals owing to the ease with which they can be bled and the quantities of blood that can be taken without affecting the animal in any way.

The blood was drawn directly from the heart in all cases. In the earlier experiments, the blood inorganic sulphate of the normal unoperated dogs was determined. It soon became evident, however, that unilaterally operated dogs are perfectly normal and can be used for controls. Later on in the work, the control blood was taken from bilaterally operated animals which did not present symptoms. This is a legitimate procedure since double-operated dogs remain normal for several days following removal of the second adrenal, and during this period abnormal blood changes do not occur. Once the animals

refuse food and seem depressed and moody, the normal period is over. Only dogs surviving the second operation from 4 to 6 days were employed in the present work. Fifteen dogs survived less than this period and were discarded for this experiment.

The method used for determining blood sulphate is that of Denis and Reed (1926) and is as follows: 25 cc. of blood was drawn directly from the heart into a 50 cc. Leur syringe and immediately placed in a small flask containing citrate. To this was added 55 cc. of redistilled H_2O, and 20 cc. of a 20 per cent trichloracetic acid solution. This was shaken well and allowed to stand for 1 hour and

TABLE I

THE INORGANIC SULPHATE OF NORMAL DOG BLOOD

NORMAL UNOPERATED		UNILATERALLY OPERATED	
Animal No.	Sulphur per 100 Cc. (Mgm.)	Animal No.	Sulphur per 100 Cc. (Mgm.)
1	0.8	12	2.4
2	1.5	13	1.6
3	0.9	14	2.8
4	1.0	15	2.5
5	1.1	16	3.1
6	1.8	17	2.3
7	1.1	18	2.1
8	2.3	19	2.2
9	0.9	20	3.2
10	1.0	21	2.1

filtered. Twenty-five cc. of clear filtrate were taken and to this were added 75 cc. of redistilled H_2O and 20 cc. of 5 per cent barium chloride. This was permitted to stand 12 hours. It was then transferred to a tarred Gooch crucible with asbestos mat, washed, dried, and ignited to constant weight. Blanks were run with all blood determinations. Merck's trichloracetic acid was used containing 0.0495 per cent sulphur. This was taken into consideration when making the calculations.

The diet of the dogs was the same before and after operation. It consisted of finely ground raw beef, bones, table scraps, and an occasional half-pint of milk. The animals were mongrels averaging 6–9 kilos in weight.

Table I shows the amount of inorganic sulphur (as sulphate) per

100 cc. of blood in normal and unilaterally operated dogs 5–7 days after removal of the right adrenal.

The unilaterally operated animals were to all appearances normal, and in no way could they be distinguished from the unoperated ones. The slight increase in inorganic sulphate of these dogs is probably of no great significance since it is within the range of error of the method employed. However, it is of interest in this connection that two other investigators recently reported a slight increase in blood sulphur of unilaterally operated dogs (Loeper *et al.*, 1926). These investigators appear not to have made sulphur determinations

TABLE II

THE INORGANIC SULPHATE OF THE BLOOD OF ADRENALECTOMIZED DOGS

ANIMAL NO.	UNILATERALLY OPERATED	BILATERALLY OPERATED	REMARKS
	Sulphur per 100 Cc. (Mgm.)	Sulphur per 100 Cc. (Mgm.)	
10	2.1	11.2	Very weak; marked acidosis
11	2.0	10.4	Weak; acidosis
12	1.7	12.7	Weak; acidosis present
13	1.4	10.2	Staggers about; acidosis
16	0.9	8.0	Animal weak; acidosis
19	1.0	11.9	Acidosis; verging on coma
22	1.1	7.2	Weak; could walk about; acidosis
23	1.8	9.1	Weak; acidosis
24	2.1	13.0	Verging on coma; acidosis

on bilaterally operated animals presenting symptoms of adrenal insufficiency.

When serious symptoms of adrenal insufficiency intervene following bilateral gland removal, the rise in inorganic sulphate becomes apparent. During the "fore" period after extirpation of the remaining gland, the interval during which the dog seems normal, no increase in sulphate is noticeable. The rise is most abrupt when the animal is verging on coma. Inorganic phosphate and inorganic sulphate behave similarly with respect to the stage of adrenal insufficiency during which the greatest increases take place. Increased concentration of phosphate and sulphate in the blood parallels the increase in severity of the adrenal insufficiency symptoms and the degree of acidosis. Table II shows the increase in blood sulphate following the onset of serious adrenal symptoms.

The rise in inorganic sulphate in the blood of double-operated dogs presenting marked symptoms is quite evident from examination of Table II. The animals were bled when they showed symptoms of acid intoxication but while they were still conscious and were able to move about. The amount of blood necessary for our determinations was considerable; and usually additional amounts were taken for pH, sugar, CO_2, and phosphorus—necessitating about 50 cc. altogether. We noted that adrenalectomized dogs presenting marked symptoms of acid intoxication rarely survived but a few hours following removal of 50 cc. of blood. The symptoms were always aggravated by bleeding. There can be no question but that bleeding shortens the life-span of adrenalectomized dogs and cats.

In several animals we were able to make determinations of the inorganic phosphorus, CO_2, pH, and sugar simultaneously with inorganic sulphate. The results are shown in Table III.

DISCUSSION

The results of this investigation show that sulphate retention occurs following bilateral adrenal removal in dogs. The rise in sulphate is especially pronounced following the onset of serious symptoms of acid intoxication, and parallels the rise in inorganic phosphate and nitrogen.

Denis (1921) reports that the inorganic sulphate of the blood of various animals varies between 1.8 mgm. and 4 mgm. of sulphur per 100 cc., whereas in normal human blood the quantity of inorganic sulphate is much less, varying from 1.0 to 0.5 mgm. S. per 100 cc. In nephritis with nitrogen retention, figures for inorganic sulphate as high as 12–16 mgm. S. per 100 cc. are not uncommon. In those types of nephritis characterized by slight nitrogen retention, the retention of sulphate is much less. It has also been demonstrated by Denis that the experimentally induced nephritis in rabbits which follows injections of large doses of uranium nitrate is characterized by marked sulphate retention. The sulphate ion is apparently excreted more slowly by the kidneys than are the other ions which constitute the normal blood salts. Consequently any malfunctioning of the acid-elimination function of the kidney mechanism leads to sulphate retention. In so far as the writers are aware, nephritis and adrenal

TABLE III

BLOOD CHANGES FOLLOWING ADRENALECTOMY IN DOGS

Animal No.	Unilaterally Operated	Sulphate per 100 Cc. (Mgm. S.)	Bilaterally Operated	First Symptoms Noticed	Survival Period (Days)	Sulphate per 100 Cc. (Mgm. S.)	pH	CO_2 Capacity (Vols. per Cent)	CO_2 Content (Vols. per Cent)	P (Mgm.)	Sugar per 100 Cc. (Mgm.)	Condition of Animal When Bled
26.....	6– 8 at 10 A.M.	0.9	6–15 at 4 P.M.	6–18 at 1 P.M.	5	11.9	7.26	27	20.6	10	56	Weak
27.....	6– 9 at 11 A.M.	1.2	6–16 at 2 P.M.	6–19 at 9 P.M.	6	10.7	7.25	25	19.2	9.2	50	Weak
29.....	5–18 at 2 P.M.	1.4	5–26 at 9 A.M.	5–29 at 10 A.M.	$5\frac{1}{2}$	11.1	7.22	22	19.0	12.1	45	Verging on coma
31.....	5–21 at 3 P.M.	1.2	5–29 at 3 P.M.	6– 2 at 11 A.M.	5	9.4	7.27	27	21.5	13.1	57	Weak
33.....	5–24 at 11 A.M.	1.2	5–30 at 9 A.M.	6– 3 at 8 A.M.	$6\frac{1}{2}$	12.2	7.25	26	21.4	10.2	62	Weak
34*....	5–20 at 10 A.M.	1.4	5–28 at 5 P.M.	5–31 at 2 P.M.	$4\frac{1}{2}$	9.1	7.27	32.1	28.1	12.5	53	Weak

* Venous blood used.

insufficiency are the only pathological conditions reported which are characterized by retention of inorganic sulphate in the blood.

It was stated earlier in this paper that previous studies on the acid-base equilibrium of bilaterally adrenalectomized cats led Swingle and Eisenman (1927) to the belief that the acid intoxication observed was due to rise in inorganic phosphate and undetermined organic acids. The sulphate ion was included with organic acid owing to the difficulty of testing for it separately. The results obtained in the present work lead us to believe that retention of the sulphate ion plays a considerable rôle in the acid intoxication of adrenal insufficiency. Whether or not the rise in inorganic phosphorus and sulphate are sufficient to account entirely for the degree of acidosis observed in adrenal insufficiency, the writers are not prepared to say. It seems likely that the fall in bicarbonate is to a considerable extent due to retention of these two acids. There is, in all likelihood, also some increase in organic acid or acids of undetermined nature.

Loeper and his collaborators (1926) report an increase of blood sulphur in three cases of Addison's disease; the proportion of the oxidized sulphur was reduced, while that of the neutral sulphur was increased. They state, furthermore, that the blood of the suprarenal artery contains much more sulphur than that of the corresponding vein and that in the artery the proportion of oxidized to neutral sulphur is lower than in the vein. They conclude that the suprarenal glands retain a part of the blood sulphur, especially the neutral.

The question of the relation of the suprarenal glands to sulphur metabolism is a promising field for investigation, but at the present time practically nothing is known concerning it. Our own experiments on sulphate retention following adrenal extirpation point to the conclusion that kidney impairment soon follows removal of the cortex leading to sulphate and phosphate retention, and they throw no light upon the rôle of these glands in blood sulphur regulation.

SUMMARY AND CONCLUSION

1. Retention of inorganic sulphate occurs following the onset of serious symptoms of adrenal insufficiency in dogs.

2. Unilaterally operated dogs and bilaterally operated animals

showing no symptoms have normal amounts of inorganic sulphate in the blood.

3. The increase in inorganic sulphate parallels the increase in inorganic phosphate and nitrogen.

4. It is probable that the retention of inorganic phosphate and inorganic sulphate is sufficient to account in large part at least for the degree of acid intoxication observed in adrenal insufficiency in dogs.

5. Organic acid is probably not so important a factor in inducing the fall in bicarbonate as the phosphate and sulphate.

6. With the exception of those types of nephritis characterized by nitrogen retention and acidosis, adrenal insufficiency is, so far as we are aware, the only other condition where sulphate retention occurs.

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AN EXPERIMENTAL STUDY OF OVARIAN REGENERATION IN MICE[1]

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THE entire question of regeneration long has held a place of prime importance as a problem for investigation. As displayed in lower forms, regeneration has been the subject of considerable experimentation; and it has been quite clearly demonstrated that many types possess a remarkable capacity for growth of new tissue or—more strictly—a redifferentiation of old tissue to subserve a new function. It is a matter of common knowledge that this is shown to a striking degree in the segmented worms, in the echinoids, and—among vertebrates—in the amphibians.

To what degree the conclusions derived from observations on lower forms may apply to mammals seems still a matter for speculation. In a general way, it has not been satisfactorily demonstrated that tissues of the higher Amniota, once differentiated, can adapt themselves to serve a different function—the plasticity displayed by lower forms evidently having been lost somewhere along the line of phylogenetic development. However, Davenport (1925), working with mice, reported a remarkable degree of regenerative capacity of ovarian tissue following ovariectomy in these rodents, regeneration occurring in 64 per cent of his animals following removal of the gonads, in many cases functional ovaries being developed. The experimental period ranged in time from 8 to 45 weeks, and he based his conclusions upon the fact that post-mortem examination revealed distinct ovarian masses on the site of the original ovaries, and in many cases embryos were found *in utero*.

Should Davenport's conclusions be correct, they would have considerable significance in their bearing upon modern ideas concerning germ-cell origin, and the question is revived: Does germ-

[1] The expenses for this investigation were defrayed by a grant from the Committee for Research in Problems of Sex of the National Research Council.

cell tissue in vertebrates arise *de novo* from the peritoneum, or is it set aside at an early stage in embryonic development, not to be duplicated or replaced later by transformation of differentiated peritoneal cells? The former idea, that of a "germinal epithelium," was advanced by Waldeyer as far back as 1870; and the latter view, one of an "early segregation," was first suggested by Nussbaum in 1880. While both of these theories have received staunch support (the former—to cite but a few—from Hoffmann, 1886; Dustin, 1907; Kuschakewitsch, 1910; von Berenberg-Gossler, 1912; Gatenby, 1916; and Essenberg, 1926; and the latter from Beard, 1900; Allen, 1906; Dodds, 1910; Swift, 1914–16; Jordan, 1917; Firket, 1920; Okkelberg, 1921, and Swingle, 1921, 1926), the question still is unsettled, with the weight of evidence in favor of the idea of an early segregation of germ-cell substance. If Davenport's report is correct, however, and regeneration of ovarian tissue from non-gonadic substance does occur in mice, such evidence would lend unequivocal support to the theory of a peritoneal origin. In his data, however, no histological evidence was advanced to show that all of the original ovary was removed in each case, and it seems essential that such evidence be established before it can be stated definitely whether regenerated ovarian tissue actually arises from the coelomic epithelium in mice, or from fragments of the original germ gland inadvertently left *in situ*.

Recently Domm (1927), in working on the problem of sex-inversion in the domestic fowl, was led to perform a large series of gonadectomies in hens. Subsequent examinations revealed in a number of instances a regeneration of ovarian substance, and these he attributed to incompleteness of removal of the original ovary, in consequence of the difficulties inherent in such an operation in the fowl. Some operations were known to be incomplete at time of removal, and regeneration was to be expected. In a few instances, however, regeneration followed apparently complete ovariotomy, despite the fact that the ovarian region had been thoroughly cauterized, the tissue regenerated being functional to such an extent as seriously to interfere with the object of operation. With regard to these latter cases Domm states (page 98) that "In such cases the mass of ovarian tissue which remained behind must have been ex-

ceedingly small—in fact, in some cases one would wager that they were so small as to be almost invisible. Nevertheless, such fragments grow and hypertrophy and counteract the effects of complete ovariotomy." In some instances onset of hemorrhage necessitated the deliberate leaving *in situ* of a small fragment of ovary. In each case this was found to undergo hypertrophy; and, although the mass left was very small, in a few months it had attained the size of a normal ovary.

The experimental work upon which the following report is based was begun with the object in view of ascertaining whether or not there is any regeneration of ovarian tissue in mice following complete ovariectomy, basing any conclusions drawn upon histological study. Such evidence would show definitely two things: (1) whether or not all of the original ovary had been removed—a factor of prime importance—and (2) whether or not the regenerated tissue actually is ovarian in nature.

The writer wishes to acknowledge his indebtedness to Dr. W. W. Swingle for his suggestion of this problem and for his advice and criticism throughout the course of the work.

MATERIAL AND METHODS

Adult female mice of healthy stock were selected for operation. While no stress was laid upon age or strain, the animals for the most part were young adults, the greater portion of them never having had litters. The operative procedure was varied somewhat, care being exercised, however, that as little non-ovarian tissue as possible be removed, in order that regeneration, should any occur, might not be hindered. In the greater percentage of cases the capsule was split and the ovary cut off at the hilus; in some instances the capsule and a short section of the stalk were included as well in removal. All extirpated material was fixed in Bouin's fluid and preserved, thus being rendered available for subsequent microscopic examination, should the need arise.

Ninety-six animals were bilaterally operated, by means of small lumbolateral incisions. Both ovaries were removed at one time, with the use of finely pointed iridectomy scissors, under a binocular dissecting microscope. Pinching off of blood vessels usually sufficed

to prevent hemorrhage, and very little bleeding occurred. The wounds were sutured by means of a single stitch in the muscle layer and in the skin; although few aseptic precautions were observed, no cases of infection occurred, and healing took place very rapidly. Anesthesia mortality, to which mice seem particularly susceptible, was practically eliminated through frequent swabbing of the throat, for which purpose toothpicks wrapped in cotton were employed. Such procedure served to check a tendency to choke through excessive salivation. The operated mice were kept on a balanced ration

TABLE I

SUMMARY OF CASES EXAMINED

Interval between Operation and Examination (Weeks)	No. Examined	No. Regeneration*	Regeneration
7	2	2	0
8	13	13	0
9	39	35	4
10	16	16	0
11	1	1	0
15	2	2	0
16	8	6	2
21	17	16	1
22	12	11	1
23	21	21	0
24	4	4	0
25	6	6	0
26	11	10	1
28	2	2	0
32	2	2	0

* Of the list in the "no regeneration" column, several showed a growth of suspicious appearing masses on the site of the original ovary; in each instance sections showed these to be non-gonadic in nature, consisting chiefly of connective and of lymphoid tissue.

(cereals, greens, milk, etc.) and were allowed to run together with unoperated females and with males.

Many of the operated animals were reopened at intervals during succeeding weeks (see Table I), and the ovarian region was examined for evidences of regeneration. All suspicious masses were removed, sectioned, and examined for traces of ovarian tissue: in a number of instances small bladderlike cysts had developed on the site of the original ovary, but sections of these revealed no ovarian tissue. Complete bilateral castration was invariably followed by atrophy of the genitalia—a condition which remained persistent.

The tubes and uterus on subsequent examination were found to be small and anemic, and the external genital parts shrunken and dry in appearance. A uterus normal in size and appearance invariably had as its sequel the discovery of a fragment of ovarian tissue, usually embedded in fat and rather heavily encapsulated.

RESULTS AND DISCUSSION

The results of the examinations are arranged in tabular form in Table I and show that, out of 96 operated cases (duplicated in many instances in the table as a result of repeated examinations), there were 9 instances in which germ-cell or ovarian tissue was discovered, indicative of regeneration. As recorded in the protocols, the histories of these regenerated cases are as follows:

OPERATION, February 6

6A. Right ovary, capsule, and some mesentery removed.

6B. Left ovary and capsule removed; ovary somewhat mutilated. Considerable bleeding.

Examined July 12 (22 weeks)

6A. Uterus and tube normal in size and appearance; a large dark mass on site of original ovary, somewhat larger than average ovary, with several whitish patches. Removed and sectioned. Ovarian.

6B. No evidence of ovarian tissue.

(Examination of original ovaries showed 6A to be deeply cut, and split in the center; 6B was entire.)

OPERATION, February 7

7A. Right ovary removed, but doubtful if entire. Capsule not removed.

7B. Left ovary removed; badly mutilated. Capsule removed.

Examined August 9 (26 weeks)

7A. Tube normal in size and vascularity; no evidence of ovarian tissue.

7B. Small mass of ovarian tissue on original site.

(Examination of original ovaries: 7A, some mutilated tissue in hilus region; 7B, ovary cut nearly in two.)

OPERATION, February 9

18A. Right ovary entirely removed, plus capsule and some mesentery.

18B. Left ovary removed, without capsule; cut off at hilus. Bad hemorrhage.

Examined April 18 (9 weeks)

18A. Very evident mass of ovarian tissue on old site. Removed and sectioned. Ovarian.

18B. A somewhat smaller mass of tissue. Sectioned. Ovarian.

Autopsied July 14 (22 weeks)

18A and B. Nothing found on either side. Uteri and tubes atrophied.

OPERATION, February 11

19A. Right ovary cut off at hilus; capsule removed, with some mesentery.

19B. Left ovary removed. Left a small lobe on one side. Cut off at hilus.

Examined April 18 (9 weeks)

19A. No evident ovarian tissue; oviduct normal.

19B. Small round fragment on site of original ovary which appeared to consist of follicles. Not romoved.

Autopsied July 12 (22 weeks)

19A. Same as the foregoing.

19B. Two fairly large white bodies embedded in fingers of tube. Sectioned and found to be typical lutein cells.

(Examination of original ovaries: 19A, entire; 19B, minus a small lobe.)

OPERATION, February 16

34A. Right ovary removed entire, plus capsule.

34B. Left ovary removed entire, plus capsule. Latter slit through at one place.

Examined April 22 (9 weeks)

34A. No evident ovarian tissue. Uterus normal in size.

34B. Adhesions; two small capsules of very evident ovarian tissue on old site, and a larger mass adherent to tube.

(Examination of original ovaries revealed: 34A, entire; 34B, with a cut on one side and a small band of protruding tissue.)

OPERATION, February 19

44A. Right ovary removed entire, cut off at hilus. Little bleeding.

44B. Left ovary removed entire. Capsule split off.

Examined April 20 (8 weeks)

44A. Adhesions; fat. No evident ovarian tissue. Uterus fairly large.

44B. Same as 44A.

Autopsied July 14 (21 weeks)

44A. Adhesions. A small mass of evident ovarian tissue deeply embedded in fat. Removed and sectioned. Ovarian.

44B. No evident ovarian tissue. Uterus and tubes normal.

(Examination of original ovaries: both appear entire, with no cuts or abrasions.)

OPERATION, December 31, Series B

BA. Right ovary removed entire, plus capsule. Cut off at hilus.

BB. Left ovary removed entire, plus capsule and some mesentery. Cut off at hilus.

Autopsied April 22 (16 weeks)

BA. Adhesions. Very evident ovarian mass embedded in fat around tube. Not preserved.

BB. Adhesions. A very small mass of ovarian tissue embedded in fat near tube. Not preserved. Tubes normal.

(Examination of original ovaries: both appear entire.)

Following the discovery of apparent regeneration, the origina ovaries were subjected to careful scrutiny, and in some cases were sectioned, to make certain that removal had been complete. Examination of original tissue in four instances (Cases 6A, 7B, 19B, 34B) would indicate that complete extirpation was doubtful—in some cases the material being somewhat mutilated. Under such circumstances, one cannot be certain that all of the original ovary had been removed; and, in view of the remarkable regenerative capacity (see below) of small bits of germ-cell tissue, it would be hazardous to assume that such regeneration had occurred from non-gonadic tissue.

Five cases remain (18A, 18B, 44A, BA, BB) of which the notes show apparently complete removal of the original ovary, examination revealing no cuts or abrasions. Such a small percentage (5 out of 96) of apparent regenerations from non-gonadic tissue gives rise to a reasonable doubt as to the nature of their origin. It seems by no means unlikely, even under the most careful operative precautions, that microscopic bits of germ-cell tissue may have been left *in situ*, particularly in the hilus region.

PARTIAL OPERATIONS

As a check on the possibilities of the regenerative capacity of ovarian tissue, a series of five partial operations was performed, to determine to what extent regeneration occurs following incomplete removal. The case histories of these are given below:

OPERATION, March 20, Series C

47. Right ovary about three-fourths removed. (Ovary rather small.) None of stalk and no mesentery removed. Some bleeding. Left ovary not removed.

Examined April 23 (5 weeks)

Right ovary average in size, surrounded by a rather heavy capsule. No adhesions.

OPERATION, March 20

48. Right ovary about two-thirds removed, in addition to most of capsule. Stalk left intact. Some bleeding. Left ovary not removed.

Examined April 23 (5 weeks)

Right side. Ovary about average size. Capsule well defined. Large blood vessels spread over capsule.

OPERATION, March 20

49. Right ovary not removed. Left ovary about one-half removed, together with half of capsule. Stalk left intact. Some bleeding. (Ovary rather small.)

Examined April 23 (5 weeks)

Left side. Ovary normal in size. Capsule rather heavier than usual, and well supplied with blood vessels.

Examined August 10 (20 plus weeks)

Right ovary removed entire. Left ovary somewhat smaller than right, and two-lobed. Follicles evident.

OPERATION, March 20

50. Right ovary removed entire. (Ovary rather small.) Left ovary two-thirds removed, plus most of capsule. Stalk and mesentery left intact. Some bleeding.

Examined April 23 (5 weeks)

Right side: no evidence of ovarian tissue; tube normal. Left side: ovary average in size; capsule normal.

Examined August 10 (20 plus weeks)

Right side: no evident ovarian tissue; tube normal in size. Left side: normal sized ovary, in normal capsule.

OPERATION, December 31

55. Right ovary and capsule removed entire. Little bleeding. Left ovary incompletely removed; a small fragment left *in situ*.

Examined March 21 (11 weeks)

Right side: no evident ovarian tissue; tube normal in size and appearance. Left side: ovary had grown into an irregular mass of tissue, with a normal capsule; red.

While these operations were only roughly quantitative, nevertheless the regenerative capacity of ovarian tissue is illustrated in a striking manner. In four cases (47, 48, 49, 50) examination after only a 5-week interval revealed hypertrophy of the tissue left *in situ* into masses approximating or equaling in size the average normal ovary: one case after an 11-week interval showed the same condition; and two cases re-examined 20 plus weeks following operation (49, 50) gave evidence of no change from the condition at 5 weeks, which would suggest that such hypertrophy is of a permanent nature.

Since the completion of this work, a paper by Parkes and Bellerby (1926) has come to the writer's attention, in which support is lent to Davenport's findings. In connection with some work on hormone injection these workers conducted a series of ovariectomies on mice; and incidental mention is made of the fact that their results, so far as regeneration is concerned, "amply confirm" those of Davenport—the criterion of regeneration being the spontaneous onset of

the oestrous cycle. The probability of regeneration, indeed, was so great that they deemed it expedient to use castrated mice as test animals for only a short time following ovariectomy. They were able, however, to reduce the percentage of regeneration to a point below that found by Davenport by "removing the whole ovarian region, including capsule and fat body, at the time of operation." How material a reduction was obtained is not revealed, but the percentage of regeneration, presumably, still remained rather high. In their work, however, apparently no check was made as to the completeness of original removal, nor is their method of operation given; and it is the writer's opinion that their original operations were not complete in all cases.

SUMMARY

1. Germinal tissue in mice will not arise *de novo* from the coelomic epithelium following ovariectomy.

2. Regeneration of ovarian tissue in gonadectomized mice does not occur during a period of 32 weeks, providing all original germ-cell tissue has been removed; and it seems highly probable that it does not occur at all.

3. The occurrence of regenerated ovarian tissue in mice following ovariectomy can be satisfactorily explained on the basis of incomplete removal of the original ovary.

4. Following incomplete castration, regeneration occurs and growth of the regenerated material takes place very rapidly; that such regenerated tissue may be functional is indicated by the fact that follicles are sometimes present.

5. Such regeneration is of a permanent nature, and is not followed by absorption of the regenerated substance.

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LOCALIZATION IN THE STARFISH EGG AND FUSION OF BLASTULAE FROM EGG FRAGMENTS

(Eight figures)

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THE starfish *Patiria miniata* is obtainable in abundance in the vicinity of the Hopkins Marine Station, Pacific Grove. California, and is sexually mature throughout the spring and early summer. Placed on a glass plate out of water, it extrudes the eggs and the sperm through the gonopores. Of 700 individuals collected in the latter part of April, in May, and in the first week of June, 33 per cent extruded eggs in the average time of an hour and a half, and 43 per cent extruded sperm in the average time of one hour. The proportion of individuals with mature germ cells increased steadily up to the first week of June, when work was discontinued. Only ripe eggs are extruded; and at the time of extrusion, the germinal vesicle has just disappeared. After about ten or fifteen minutes in sea-water the first polar body appears.

The eggs are large and quite transparent. When spherical, they are 180–90 microns in diameter, exclusive of the jelly. Very often, when freshly laid, they are not quite spherical, but are shorter in the polar axis, which is about 165 microns, while the longer axis is about 220 microns. The eggs cleave normally if fertilized within about 2 hours at laboratory temperature (17° C) or 3 hours at 11°, which is the temperature of their natural environment. The range of temperature of the shore water of Monterey Bay in the spring is about 10°–12.° When eggs older than 3 hours were inseminated, fertilization membranes developed, but the eggs fragmented. A small percentage of eggs even 24 hours old developed very weak membranes. *Patiria* eggs are especially sensitive to temperature, and egg fragments are even more so. Fragmentation was almost entirely eliminated by frequent change of water dipped from the Bay in pyrex

vessels and by rearing the embryos in a constant-temperature box kept at 11°. By this treatment 95 per cent of controls were repeatedly reared to the gastrula stage. At laboratory temperature (17°), however, complete or partial fragmentation was frequent.

One female produced a number of double-sized eggs, slightly bilobed, with a single nucleus. These underwent normal development, producing double-sized gastrulae, with double-sized cells. Giant eggs of sea urchins and other animals have been observed by a number of workers.

In all, 181 eggs were cut in two parts with quartz micro-needles in a moist chamber under 120 diameters magnification by means of a Taylor micro-manipulator (Taylor, 1925). Each fragment was reared in a small individual glass dish. The polarity of these eggs can readily be recognized by the position of the large and conspicuous polar bodies and by the position of the egg nucleus which lies close under the polar bodies for about an hour after maturation. The nucleus gradually drifts, at a rate varying with temperature, to a position about two-thirds of the way from the pole to the center of the egg and remains there until the egg is fertilized or disintegrates.

The eggs were cut in some cases along the polar axis, in other cases perpendicular to the polar axis. In fragments from all types of cuts, with rare exceptions, the first two cleavage planes are perpendicular to the cut surface. This had been previously observed in *Patiria* by Taylor and Whitaker (1926), and in the sea urchin *Lytechinus* by Taylor and Tennent (1924). Harnly (1926) reports that in *Arbacia* eggs cut between 5 and 6 minutes after fertilization, and at random with respect to the polar axis, the first two cleavages of both fragments are perpendicular to the cut surface.

In *Patiria* there is nearly always an infolding of the early blastula wall on the cut surface. This occurs whether the fragments are completely isolated or are left in the same fertilization membrane (a condition which will be described later), and regardless of the plane of section (Fig. 1). This infolding was described by Taylor and Whitaker (1926) and was regarded as "evidence, yet hardly complete, that the original polarity of the egg does not necessarily persist in the fragment." That the original polarity does not necessarily persist in the fragments of eggs of the sea urchin *Lytechinus*

(*Toxopneustes*) *variegatus* was indicated by the occurrence of micromeres at the end of an axis perpendicular to the cut surface, in fragments of eggs cut in various planes. (Taylor and Tennent, 1924; Taylor, Tennent, and Whitaker, 1926). In the fragments of *Patiria*, however, it does not now appear that the infoldings on the cut surface referred to can be regarded as true archentera; for detailed studies on some 90 fragments have shown that although the inturned wall is usually about the size of an archenteron or slightly smaller: (1) it appears at an earlier stage than does the archenteron of the control, while the development of fragments is somewhat

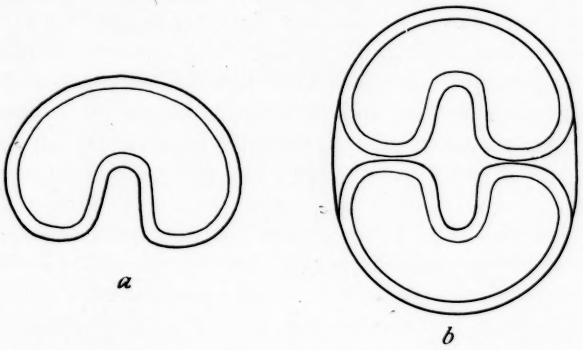

FIG. 1.—Early blastular infolding on cut surface (*a*) of blastula from egg fragment, (*b*) of two blastulae from two fragments within the same fertilization membrane.

slower (the inturning often appears as early as ten to sixteen hours after fertilization, while in the normal egg at the same temperature [11°] the archenteron does not develop for forty-five to forty-eight hours); (2) the inturning is not preceded by thickening, nor is it accompanied by proliferation of mesenchyme, as is the archenteron of the normal egg; (3) the inturning is not permanent, but in healthy fragments it pushes out and becomes part of the blastula wall after 5–10 hours, and a day or a day and a half later a true archenteron, with thickening and mesenchyme, develops.

An extended effort was made to determine if this second invagination occurs on the site of the first infolding, that is, on the cut surface. Until the early blastula stage is reached, a flattening of the

embryo marks the cut surface; but when the blastula becomes free-swimming, it changes its shape; it rounds up and swims for about 24 hours before the thickening which precedes the true archenteron develops. Ninety fragments were followed, some with almost uninterrupted observation. A few fragments had slight protrusions which served as markers of the cut surface; but it was found impossible to make out whether there is any relation between the locations of the early inturning and the archenteron.

Newman (1921) describes blastulae of *Patiria miniata* having a retarded rate of development which become twins by a multiple gastrulation, as a result, he concludes, of the elimination or radical disturbance of the axial gradient or general polarity. From his drawings and description the invaginations appear to be similar to the early inturning described above. That they are archentera seems questionable. The same sort of blastulae are also produced by forcing the eggs either before or after fertilization through a tightly fitting glass capillary. Fragments which are squeezed excessively in cutting often develop more than one such infolding. In most of these cases, however, if the embryos recover and become healthy, the inturnings push out and disappear. Not uncommonly two or even more independent blastulae result from a pinching off of parts of the original blastula. Often these fuse at a later time, giving various sorts and degrees of twinning.

Whether the early inturning on the cut surface represents a change of polarity, or a temporary change, or tendency to change, is at present perhaps a matter of interpretation.

DEVELOPMENT OF FRAGMENTS FROM CUTS PERPENDICULAR TO THE POLAR AXIS

Sixty-one eggs were cut along the equator giving equal-sized fragments which were reared under observation from five to ten days. In eight cases both fragments of a pair either failed to be fertilized or disintegrated too early to yield a result. In fourteen cases the polar[1] (nucleated) fragment developed and the antipolar disin-

[1] The improved terminology is here adopted, in which the point at which the polar bodies are given off is called the "pole," and the other end of the polar axis is called the "antipole."

tegrated. In three cases the antipolar fragment developed and the polar disintegrated. In thirty-six cases both fragments developed for at least five days. The results were as tabulated in Table I.

In all cases there was a marked difference between the embryos from a pair of these fragments. The degree of difference varied; but in all cases of healthy development of both, the antipolar fragment showed a greater tendency to gastrulate. Thus of the thirty-nine reared antipolar fragments, twenty-four became gastrulae, while the other fifteen developed thickenings or shallow invaginations. Of the 50 reared polar fragments, 34 became blastulae only, while the other 16 developed slight thickenings of the blastula wall. But these

TABLE I

EGGS CUT ALONG THE EQUATOR

Cases	Polar Fragments	Antipolar Fragments
13	blastula only	gastrula
11	blastula only	disintegrated
10	blastula only	blastula with thickening, or with shallow archenteron
9	blastula with slight thickening	gastrula
4	blastula with slight thickening	blastula with thickening, or with shallow archenteron
3	blastula with slight thickening	disintegrated
2	disintegrated	gastrula
1	disintegrated	blastula with thickening
8	disintegrated	disintegrated

thickenings were not as heavy as those of the antipolar blastulae that failed to form complete archentera. The polar blastula is comparatively thin walled, active, and swims high in the water (Fig. 2*a*). The antipolar blastula is very thick walled, inactive, and swims slowly on the bottom (Fig. 2*b*); it is also less viable. The cells of an embryo from an antipolar fragment appear to be gorged with yolk, and it seems probable that the differences between polar and antipolar fragments are at least in part due to the higher concentration of yolk at the antipolar end of the egg.

The embryo from the polar fragment of the equatorial cut does not develop an archenteron; but in its place at about the time the archenteron develops in other fragments (i. e., about fifty hours after insemination), a slight infolding occurs, usually around a small pore that appears at this time in the blastula wall (Fig. 2*a*). Often a

moderate thickening occurs before this, varying in degree in different eggs. Mesenchyme is proliferated around the pore from this same region. After a few hours this pore closes, such infolding as has occurred disappears, and the blastula becomes uniform and well rounded. In 6 cases mesenchyme was at first proliferated outward through this pore; but as soon as the pore sealed, new mesenchyme was proliferated on the inside of the blastula wall. Several hours after the proliferation of mesenchyme from around the pore, a proliferation of mesenchyme begins at the opposite end of the blastula, usually preceded by a localized thickening which has the character-

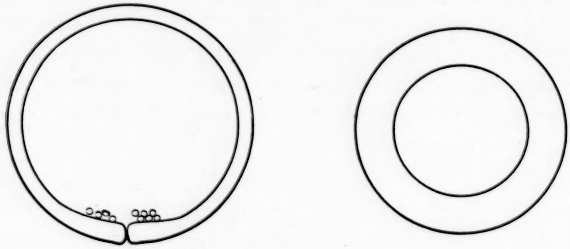

FIG. 2.—(*a*) Blastula from polar half of egg showing thin wall, pore which forms in place of archenteron, and mesenchyme proliferated about the pore; (*b*) blastula from antipolar half of egg showing thick wall due to heavy content of yolk.

istic shape of the apical plate (Fig. 3*a*). This ectodermal organ is unique in *Patiria*, among starfishes, and is a source of mesenchyme in the late gastrula stage of the normal larva (Heath, 1917). Many of the blastulae were kept from fifteen to twenty days. They finally became very thin walled, and died without further advances in development beyond multiplication and distribution of mesenchyme.

In an embryo from the antipolar fragment (Fig. 3*b*), the blastula wall thickens and invaginates to form a large and massive archenteron, larger in proportion to the size of the fragment than that of the normal egg. Mesenchyme is proliferated mainly from pouches on the sides of the archenteron. The embryo is heavier and less active than the embryo from the polar fragment. The blastocoele of the gastrula is small, owing to the large size of the archenteron and the

thickness of the body walls. Not as much mesenchyme is proliferated in the antipolar as in the polar embryo. However, there is less protoplasm in this fragment, since it contains more yolk. A stomodaeum does not appear, and in ten or more days the embryo disintegrates. It is not uncommon for the archenteron to disintegrate during the later days of the embryo, causing, in a sense, a reversion to the blastula stage. In several cases after this had happened, a second archenteron developed, on the site of the first.

Another experiment was made to see if the localization in the egg, which causes the differences between polar and antipolar fragments,

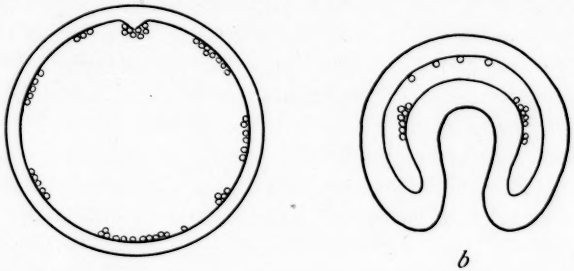

FIG. 3.—(*a*) Blastula from polar half of egg 3 days old showing disappearance of pore in wall, and showing apical plate, with proliferation of mesenchyme; (*b*) gastrula from antipolar half of egg showing massive archenteron, larger in proportion than in the normal egg.

undergoes change during the period between maturation and a time when the egg is too old for normal development. This period is two to three hours long. Twelve eggs from three females were cut along the equator after resting in sea-water from 2 to $2\frac{1}{2}$ hours after maturation, and then inseminated. Ten polar fragments became blastulae only, 2 disintegrated; 5 antipolar fragments became gastrulae, 7 disintegrated. As a control 10 eggs from the same three females were cut after the same fashion immediately after maturation, and inseminated, with the usual result. Ten polar fragments became blastulae; 7 antipolar fragments became gastrulae, and three disintegrated. The results show no detectable change in the egg during this time, as regards this localization.

Eggs were also cut perpendicular to the polar axis into pieces of

unequal size. Eight eggs were cut so that the polar fragment contained about one-fourth of the volume of the egg; the antipolar, three-fourths. The 8 polar fragments became blastulae, only 1 with a slight thickening. Six of the antipolar fragments developed deep archentera, 2 developed shallow archentera. Mesenchyme formed in all. Four eggs were cut so that the polar fragment contained about three-fourths of the egg; the antipolar, one-fourth. Three polar fragments developed shallow archentera, one became a blastula only. The 4 antipolar fragments all developed deep archentera. These antipolar fragments developed less mesenchyme than the other fragments. The results are consistent with those from equal-sized fragments. The greater the proportion of material from the antipolar end of the egg, the larger and deeper the archenteron.

Fertilized nucleated fragments are more viable than nonnucleated. Furthermore, if fragments are allowed to stand some time after cutting before insemination, it is found that nucleated fragments can be fertilized after a longer period than nonnucleated fragments.

The first division of either fragment after an equatorial cut gives two equal-sized cells, while the first division of fragments from a cut along the axis gives one larger and one smaller cell. The division spindle is shifted away from the antipolar end.

If unfertilized eggs are forced through a glass capillary which stretches them to 3 or 4 times the length of the normal diameter, the cortex splits and the interior cytoplasm runs out, leaving a cortical husk. Chambers (1921*a*) pressed out the interior cytoplasm of the starfish egg through a cut made by a glass needle and found that the cortex could be fertilized and would undergo cleavage. Five *Patiria* "husks" produced small gastrulae. There is a certain amount of endoplasm left inside the "husk," but probably none of it comes from the central region of the egg.

Chambers (1921*b*) concludes from micro-dissection experiments that the fertilization membrane in the starfish (as well as in other Echinoderms) exists as a distinct morphological structure before fertilization. He states that it can be lifted off the unfertilized egg by means of the micro-needle. The same is true of the eggs of *Patiria*. When an attempt is made to cut an egg in two with a micro-needle, it is found that the cytoplasm is easily squeezed into two parts but

that it is much more difficult to sever the tough outermost membrane. If it is not severed, and if the divided egg is inseminated, both fragments are fertilized and develop independently within the same fertilization membrane. In the early blastula stage (about 128–256 cells) the two blastulae will often fuse. If they do not fuse, they behave like embryos from fragments which had been completely separated before fertilization. The stage at which the two blastulae fuse can be determined to some extent by the degree to which the fragments are pulled apart before fertilization, that is, by the extent to which the outer membrane is stretched. It is elastic, but when stretched, comes back only part way. Even when the two groups of cells are closely pressed together, they do not adhere before the early blastula stage.

Seven cases were observed in *Patiria* in which the independently fertilized fragments within the same fertilization membrane reunited and fused before the first cleavage. In all these cases they separated again at the first cleavage, which produced 4 cells; and the 2 fragments developed independently into blastulae.

Thirty-two eggs were cut along the equator, with the outer membrane unsevered, so that the 2 fragments came to lie within the same fertilization membrane. Twenty-four pairs fused in the blastula stage. The result is an embryo which is more viable than either of the halves when developed separately. The embryo is at first bilobed (Fig. 4). The antipolar lobe is smaller, and its walls are much thicker than those of the polar lobe. Gradually the shape of the embryo becomes more like the normal, finally becoming almost exactly like it, in spite of the fact that some of the cells are haploid, some diploid.

The antipolar half cleaves more slowly than the polar half, in the early stages at least. That this is due both to its being haploid and to its containing more yolk, or at least some material from the antipolar region of the egg, is indicated by the following experiment: Ten eggs were cut along the polar axis, each giving 2 equal-sized fragments, 1 containing the egg nucleus, the other not. After fertilization, one fragment is haploid, the other diploid; otherwise the fragments are presumably identical. The diploid fragments undergo the first cleavage from five to ten minutes before the haploid, and lead

in rate of cleavage at least throughout the early stages. Ten other eggs were cut along the equator, giving two equal halves. The antipolar halves, when fertilized, are haploid. When compared with the haploid fragments from the polar-axis cuts, they cleave more slowly. This indicates that the materials near the antipole decrease the rate of cleavage. The higher concentration of yolk is probably the effective agent.

FIG. 4

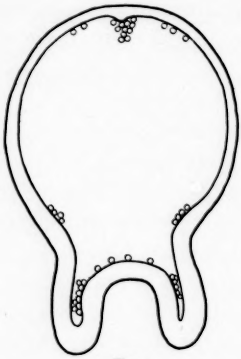

FIG. 5

FIG. 4.—Embryo resulting from fusion of blastulae from polar and antipolar fragments, showing antipolar component with thicker walls and with archenteron. Mesenchyme is shown proliferated from archenteron, from posterior end of polar component, and from apical plate.

FIG. 5.—Later stage of Figure 4. Wave of thickening of gastrular wall has spread forward into polar component.

In the fused blastulae from the equatorial cut, the primary cavities unite and are shared in common. The archenteron develops on the lobe from the antipolar fragment at the place where it would have developed had the egg not been cut. Since the blastulae are flattened on the adjoining sides, they usually fuse together in exactly the same relationship that the parts of the egg had before cutting. Sometimes there is a slight rotation of one of the blastulae, in which case the archenteron is partly on the side (Fig. 6).

Mesenchyme is proliferated both from the archenteron of the posterior component and from the posterior rim of the anterior component (Fig. 5). The proliferation in the anterior component is at

the same point at which it would have developed in the polar blastula had that not fused with the antipolar blastula. Mesenchyme also comes from the apical plate at a later time. These embryos live more or less indefinitely and appear to undergo approximately normal development.

It has been stated that the part of the embryo which is derived from the antipolar fragment has thicker walls. At the time when the archenteron has begun to invaginate, or soon thereafter, there is a forward progression or wave of thickening which extends into the thin-walled anterior part. This results in a thinning of the thick posterior walls and a thickening of the thin anterior walls, i. e., the walls become more uniform throughout. If the two lobes are cut apart at the constriction where they grew together, they both close in, and both pieces then round up. Five of these embryos were cut apart before the thickening had spread forward. Each of the posterior parts, derived from the antipolar fragments, developed an archenteron; the anterior parts did not. Five others were cut apart after the wave of thickening had spread into the posterior part of the anterior lobe (Fig. 5). Each of the anterior parts, as well as the posterior parts, developed an archenteron.

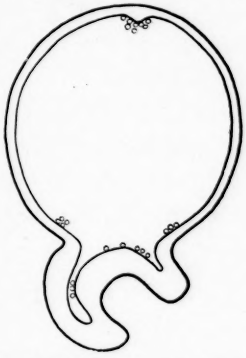

FIG. 6.—Embryo resulting from fusion of blastulae from polar and antipolar fragments when blastulae have rotated slightly, so that the axes are no longer continuous.

After the cutting, the anterior lobe draws in and closes the hole which was made by cutting through the primary cavity. This results in a bunching of the thickened parts at the region of closure, and the archenteron develops as an ingrowth of this thickening. Mesenchyme is given off from the archenteron and later from the apical plate. In all 5 cases an archenteron developed; but it was not as deep as is the normal archenteron, and the embryos disintegrated before a stomodaeum developed. The gastrulae from the posterior lobes also disintegrated before a stomodaeum developed.

DEVELOPMENT OF FRAGMENTS FROM EGGS CUT ALONG THE POLAR AXIS

Thirteen pairs of equal-sized fragments from cuts along the polar axis, and 8 more fragments from pairs of which one member did not develop produced embryos which, except for size, were normal and complete. The fragments which contained the egg nucleus, and

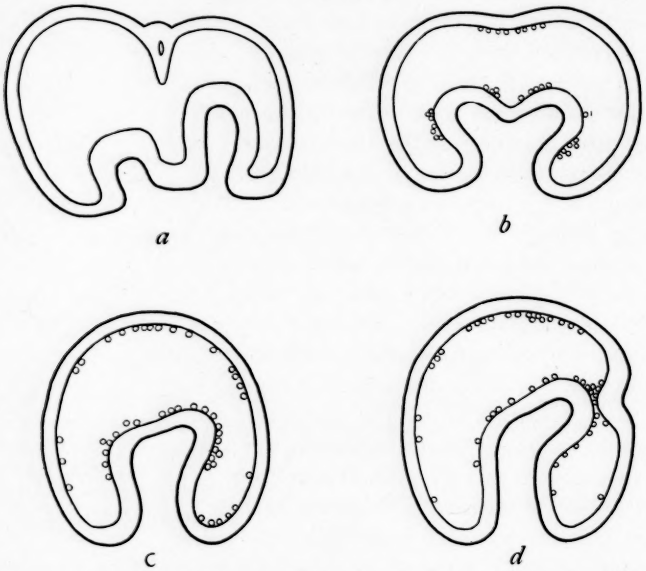

FIG. 7.—Four stages in adaptive development of a twin resulting from late fusion of two blastulae from fragments coming from an egg cut along the polar axis; (*a*) 2½ days old, (*b*) 3½ days, (*c*) 4½ days, (*d*) 8 days.

which were diploid after fertilization, usually cleaved sooner and were first to gastrulate.

Thirty eggs were cut along the polar axis, with the outer membrane unsevered, so that the fragments came to lie within the same fertilization membrane. In 22 cases the resulting pairs of blastulae fused. The other 8 pairs either disintegrated and were discarded, or failed to fuse and developed as independent embryos. Of the 22 cases of fused blastulae, 13 pairs fused in the late blastula stage. An

archenteron developed on the antipolar end of each lobe. Usually one archenteron developed earlier than the other and kept ahead until the archentera fused, which they did after gradually coming together. A certain amount of material was sometimes sloughed off into the primary cavity. One well-formed archenteron resulted. Mesenchyme was first proliferated from each archenteron as it started to invaginate, and later from pouches on either side. In case of late fusion of the blastulae, the union of the archentera is delayed so that the head of the archenteron is still double at the time of the formation of the pouches, which are partly or completely paired. The duplicate pouches finally fuse, however. Later mesenchyme develops at the apical plate. The embryo gradually assumes the shape of a normal gastrula. Four of the 13 developed a single stomodaeum (Fig. 7). The others lived for several days but disintegrated without forming stomodaea. By this time the twin characteristics have been almost entirely eliminated.

The other 9 pairs of blastulae within the same fertilization membrane from cuts along the polar axis fused at about the earliest stage at which they will grow together, that is, at about the 128- or 256-cell stage. A single archenteron developed on only 1 lobe. The archenteron was inhibited in the oth,er but at the place where it would have developed had the blastulae fused at a later stage, a thickening occurred with a slight proliferation of mesenchyme (Fig. 8). The degree of inhibition of the second archenteron varied with the stage at which union of the blastulae took place, and also with the degree of their association, that is, with the extent to which they were united.

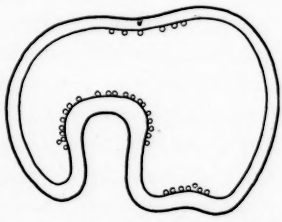

FIG. 8.—Embryo resulting from early fusion of two blastulae from fragments from an egg cut along the polar axis. This shows the earlier adaptation. (Compare Fig. 7*a*). One archenteron forms first and practically inhibits the other.

When one of two lobes of fused blastulae which have come either from 2 egg fragments or from isolated blastomeres within the same fertilization membrane, develops more slowly than the other, or begins to disintegrate, the bulk of its substance is taken over and incorporated into the healthy

lobe. The cells that are not themselves disintegrated move over to the healthy lobe, and this lobe becomes proportionately larger. Its own shape and proportions are affected only temporarily. In other cases only some of the cells were incorporated, after which the backward lobe recovered. The result was a twin with lobes of unequal size. But eventually the smaller was taken over by the larger. More than 20 cases of various sorts and degrees of this incorporation were observed. Driesch has recorded cases of predominance of one individual in fused sea-urchin blastulae.

DISCUSSION

LOCALIZATION

Boveri (1901) concluded that in the sea-urchin *Paracentrotus lividus* the micromeres and primary mesenchyme are derived from material that is localized before fertilization in a cap at the antipolar end of the egg. It was generally inferred from this that an egg fragment could produce micromeres only if it included some of the antipolar material. Taylor and Tennent (1924) found in *Lytechinus variegatus* that polar as well as antipolar hemispheres of eggs cut before fertilization produce micromeres as well as mesenchyme and spicules. Both embryos are normal except for size.

Harnly (1926) suggests that in *Arbacia* before fertilization there is a localization of micromere-forming material that lies between the nucleus and the geometric center of the egg. Only fragments cut so as to include some of this material form micromeres. Taylor, Tennent, and Whitaker (1926) cut a large number of *Lytechinus* eggs by the micromanipulation method and found that small fragments cut from any region of the egg, either with respect to the polar axis or with respect to the position of the nucleus, develop micromeres and mesenchyme.

At the pole of the egg, however, there appears to be a cap consisting largely of ectoderm-forming material. It is only about one-tenth the volume of the egg. Small fragments from the pole of the egg are not able to gastrulate, whereas equal-sized fragments from any other region form gastrulae. Also less mesenchyme is developed from the small polar fragment. A detailed consideration of the evi-

dence on this subject is contained in a recent publication by Tennent, Taylor, and Whitaker (Carnegie Pub., in press).

The egg of the starfish *Patiria miniata*, in contrast to that of the sea urchin, has a marked concentration of yolk at the antipolar end of the egg. Therefore the polar fragments contain much less yolk than the antipolar. And since the egg nucleus lies near the pole, the polar fragments are diploid when fertilized, while the antipolar are haploid. This results in a constant difference between these two fragments which does not exist in the sea urchin, since the egg-nucleus lies at random. But in the localization of organ-forming materials, the two eggs (*Patiria* and *Lytechinus*) are almost alike. In both mesenchyme develops in fragments from any part of the egg (with slight exception noted in *Lytechinus*). In both there is a localization of entoderm-forming material toward the antipole of the egg. But in the starfish this entoderm material is much more sharply concentrated, while it is more dispersed in the sea urchin. In the starfish it is contained almost entirely in the antipolar hemisphere, or even the antipolar one-fourth of the egg; while in *Lytechinus* it is absent from only about the polar one-tenth of the egg.

Von Übisch (1925) found that polar halves of sea urchin (*Echinocyamus pusillus*) blastulae cut along the equator did not gastrulate, while antipolar halves did. In the blastula stage, then, the sea urchin has developed a localization of entoderm material in the antipolar hemisphere, comparable to the localization which already exists in the unfertilized egg in *Patiria*.

The material or materials of the antipolar end of the *Patiria* egg, which are necessary for the development of an archenteron, apparently retain their localization and their function until at least the gastrula stage. In the fused blastulae from polar and antipolar fragments, just after gastrulation there is a forward shift of entoderm, for, if the polar part is cut off, it gastrulates only when some of this thick walled layer is included. Until this stage, the organism has been bi-lobed. It then gradually rounds out, and it is perhaps partly as a consequence of this reshaping that the entoderm moves forward. Von Übisch (1925), after inducing a forward growth of entoderm in *Echinocyamus* (by subjecting blastulae to the action of lithium, which increased the proportion of entoderm compared with

ectoderm, as Herbst had done earlier), cut the resulting blastulae into two parts. Polar halves cut from these blastulae developed shallow archentera, showing that the entoderm had been extended above the equator; while, as has been noted, polar halves of *Echinocyamus* blastulae which had not been treated with lithium did not gastrulate.

FUSED BLASTULAE

Morgan (1895) found fused blastulae in the sea urchin *Sphaerechinus*. Two archentera usually developed; but if near each other, they grew into one. Often the archenteron of one component developed first and seemed to inhibit the other. Driesch (1900, 1910) brought about the fusion both of *Echinus* and of *Sphaerechinus* eggs. The former fused more readily. In both these species he found a number of types resulting from the fused eggs: (*a*) normally proportioned embryos with double-sized archentera due to a double number of normal-sized cells; (*b*) partly reorganized embryos with 2 archentera, but with approximately normal shape or approximately normal skeleton; (*c*) twins with partial or complete independence of organs. In some cases the two components were unequal in size and in the degree of their participation in the development. In *Sphaerechinus* he also found cases of a small "parasitic" archenteron.

In 1895 and 1909 Loeb produced sea-urchin twins in different ways. He placed fertilized *Arbacia* eggs in dilute sea-water. The absorption of water burst the fertilization membrane and caused an outpouring of some of the cytoplasm. The cytoplasm that had protruded and that which remained inside the membrane segmented independently. Later the two parts fused as blastulae and formed a twin. In 1909, by the use of certain mixtures of antagonistic salts, Loeb caused the two blastomeres of the two-cell stage to shrink apart and independently develop into blastulae. These blastulae often fused and produced twins.

Bierens de Haan (1913*a*) found that by adding alkali to sea-water he could cause unfertilized *Arbacia* eggs to fuse. Fertilized eggs or blastulae were made to fuse by using alkali and Ca-free sea-water. When two eggs fused before fertilization, a giant embryo resulted, with the normal number of double-sized cells. When they fused after fertilization, a giant embryo resulted with a double number of

normal-sized cells. The fused larvae were often defective, especially in the skeletons. In many cases two archentera were formed, making various angles with each other. In some cases they fused and became one. When two blastulae had fused together at the antipoles, both became exogastrulae. Bierens de Haan suggested hypothetically that a unified organism resulted only when the two blastulae were fused in symmetric relationship, with axes parallel, that is, with relative positions similar to those of two normal blastomeres.

The blastulae of different species (*Parechinus microtuberculatus* and *Paracentrotus lividus*) were also caused to fuse by using the centrifuge in addition to Ca-free sea-water and alkali. In some cases each component produced its own type of pluteus; in a few cases some evidence of mutual influence resulted (Bierens de Haan, 1913*b*).

Goldfarb (1913) fused *Arbacia* eggs by the same method. Fusion usually took place in the blastula stage or later. He found true twins; partial twins with common blastocoele, or with partly fused archentera; and complete fusion with a single set of organs. Later, while working at Tortugas, Goldfarb (1914) found that the addition of isotonic NaCl solution to sea-water also caused blastulae to fuse. Fused blastulae of *Lytechinus* (*Toxopneustes*) gave results similar to those of *Arbacia*, except that in *Lytechinus* the skeletons of the component parts did not combine, whereas in *Arbacia* they did (Goldfarb [1915]).

Von Ubisch (1925) fused eggs of the Norwegian sea urchin *Echinus miliaris*. After shaking off the fertilization membranes, he placed eggs in Nile-blue sulphate. As soon as they had become deeply stained, he replaced them in fresh sea-water. As cleavage began, he placed the eggs in Ca-free sea-water along with similar eggs which had not been stained. Random fusions of eggs and of groups of blastomeres, stained and unstained, took place. With the aid of a glass needle, particular fusion groups were separated out and followed. Since the cells coming from a stained egg kept their color, the origin of later cells could be accurately determined in the case of a stained egg fused with an unstained egg. He found the usual types of twins and partly or wholly unified organisms. Unity occurred only when half the archenteron, half the mesenchyme cells, and half

the ectoderm were from each egg. From this he concludes that it is a morphological necessity that the axes be parallel in these cases and that the hypothesis of Boveri and De Haan is correct: namely, that unity results only when the axes of the fused blastulae are parallel.

The blastulae from fragments from cuts along the polar axis of *Patiria* eggs are normal except for size. When fused, their behavior is very similar to that of the fused blastulae from whole eggs of sea urchins, according to the accounts of other investigators. When they are fused with axes parallel, a unified organism results, as Beriens de Haan suggested. But the organism becomes unified only after a period of readjustment. The amount of readjustment depends on the stage at which fusion has taken place (compare Figs. 8 and 7*a*). The readjustment of an embryo with two archentera is shown in Figure 7.

The blastulae resulting from fragments from cuts along the equator are not equivalent to normal blastulae. When they are fused with axes continuous, never more than one archenteron is formed. The readjustment necessary is less, and the two parts readily become unified. There is a slight persistence of the dual origin in the proliferation of mesenchyme in the polar component at its antipolar end —that is, near the region of fusion with the antipolar component even though the components were fused some time before the development of mesenchyme. However, mesenchyme comes readily enough from different primitive tissues of *Patiria*, as evidenced by the normal proliferation from the ectodermal apical plate as well as from the archenteron.

Newman (1921) describes various sorts of early twinning in mass cultures of *Patiria* eggs, some as the result of partial isolation of blastomeres, some as a consequence of the elimination or radical disturbance of the axial gradient and general polarity of blastula or gastrula. He shows that there is a definite correlation between twinning and retarded development. In this connection it may be pointed out that the conditions which cause retarded and unhealthy development also increase sloughing off, and separation of the cells in the cleavage stages so that they form independent blastulae, which, since they occur within the same fertilization membrane, may often fuse together at various stages. Various types and degrees of twin-

ning result from such fusions. It does not seem to me that there is reason to believe that the twins are produced in any other way than by the fusion of independent individuals. Newman describes gastrulae with bifurcated archentera, some with duplication of mesenchymal pouches. These, he concludes, result from a splitting of the axial gradient. In the present work some thirteen such twins have been produced by cutting normal eggs along the polar axis and allowing the resulting blastulae to fuse. Two archentera form and gradually grow together. This process has been followed day by day in each individual, and the double-headed archenteron is found to be a late stage in the fusion of the archentera (Fig. 7). The archentera do not split and become twinned. As soon as the two organisms are united, duplications tend gradually to be eliminated.

SUMMARY

1. Fragments cut before fertilization from any part of the egg of *Patiria miniata* develop into embryos with mesenchyme. In this respect the egg of *Patiria* is like that of the sea urchin *Lytechinus variegatus*.

2. Fragments from the polar hemisphere develop into blastulae with mesenchyme proliferated both from the polar apical plate and from the antipolar end. They do not develop archentera. These fragments contain less yolk than the normal amount, and the blastulae are thin walled.

3. Fragments from the antipolar hemisphere develop into gastrulae with a proportionately large archenteron and with mesenchyme. These fragments contain more yolk than the normal amount, and the blastulae are thick walled.

4. The two halves resulting from a cut along the polar axis of the egg develop into complete normal gastrulae, except that they are small. The diploid embryo develops slightly more rapidly than the haploid.

5. If two blastulae lie within the same fertilization membrane they often grow together and have a primary cavity in common. When they come from egg fragments, they are usually flattened on the side of contact and grow together in the same relative positions as the original fragments of the egg, producing what is at first a bilobed embryo.

6. If the blastulae from a polar and an antipolar fragment grow together in this way, an archenteron develops on the lobe derived from the antipolar fragment.

7. The antipolar lobe is thick walled; the polar, thin. At about the time of gastrulation a wave of thickening passes forward from the antipolar to the polar lobe.

8. If these lobes are cut apart before this thickening wave has passed forward, the antipolar blastula develops an archenteron; the polar does not.

9. If the lobes are cut apart after the thickening wave has passed forward, the polar as well as the antipolar blastula develops an archenteron.

10. If two blastulae from fragments resulting from a cut along the polar axis grow together as late blastulae, an archenteron develops in each lobe. The two archentera grow together, fuse, and become one. One stomodaeum develops.

11. If two such blastulae grow together at an early stage, the archenteron forms in one lobe only and is inhibited in the other.

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THE RESPIRATORY REGULATION OF THE CRAYFISH, CAMBARUS IMMUNIS (HAGEN)

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INTRODUCTION

THE possible influence of the oxygen tension in the environment on the oxygen consumption of animals has interested many observers. The range of organisms studied has been considerable, and a brief survey of the types studied and results obtained is appropriate to the results described in this paper. Thunberg (1905), working on the air-breathing invertebrates (*Limax*, *Tenebrio*, *Lumbricus*), reported a partial dependence of oxygen consumption on the oxygen tension present in the environment. Henze (1910) in a comparative study of low and high invertebrates found that in the former (*Anemonia*, *Sipunculus*, *Actinia*) the dependence upon oxygen tension of the water was very marked in that oxygen consumption decreased as the oxygen tension was lowered. Conversely, the same author reports a relative independence of the oxygen consumption on the tension present for some of the higher invertebrates (*Eledone*, *Scyllarus*, *Carcinus*, *Aplysia*). On the basis of these findings, Henze drew the general conclusion that with the exception of some of the simpler organisms, the metabolic rate of cold-blooded animals is quite independent of the oxygen tension of their environment. This he interpreted as being due to the presence of adequate respiratory or circulatory systems whereby the oxygen tension of the various tissues remained unchanged. In the case of the lower invertebrates (*Anemonia*, *Sipunculus*, *Actinia*) the dependence on oxygen tension was explained as due to the lack of respiratory regulation, whereby the oxygen tension of some of the tissues is greatly reduced, perhaps even to zero. Krogh (1916) accepts, in general, Thunberg's explanation, believing that the lower invertebrates under question are provided with relatively imperfect respiratory systems and hence respiratory regulation under different

environmental oxygen tensions. McClendon (1917), working on *Cassiopea*, reports a lack of respiratory regulation in that lowering of oxygen tension of the water tends to lower the oxygen consumption. Lund (1921) likewise finds that in *Planaria* a dependence of oxygen consumption on the oxygen tension present is evident, although only after the oxygen tension has been reduced below the one-third saturation point of the water. *Paramecium*, on the other hand, according to the same author (1921), is quite independent of the oxygen tension present over a considerable range. More recently, Amberson, Mayerson, and Scott (1924–25) have shown that the oxygen consumption of some of the higher marine invertebrates (*Homarus americanus*, *Nereis virens*) is very closely dependent on the oxygen tension of the water over a rather wide range. In fact, the oxygen consumption at any point is directly proportional to the oxygen tension of the sea water at that instant. Preliminary experiments by the same workers give evidence that the same holds true for the king crab (*Limulus polyphemus*) and for the blue crab (*Callinectes sapidus*). The shrimp (*Palaemonetes*), however, was found to possess respiratory regulation down to about 50 per cent saturation; while the squid (*Loligo pealei*) was able to regulate its oxygen consumption down to about 30 per cent saturation. Below 50 per cent saturation, however, the squid (*Loligo pealei*) was found to lose its power of respiratory regulation, the amount of oxygen consumed being then proportional to the oxygen tension present. Amberson *et al.*, conclude, therefore, that Henze's distinction between the simpler and complex invertebrates as regards their dependence on the oxygen tension present cannot be regarded as absolute, since their data show a decided lack of respiratory regulation for certain complex invertebrates which possess well-developed respiratory systems. Their further observation that no general law can be made in this regard relative to the higher invertebrates is shared by the writer in view of the results to be described in the present paper.

It seemed profitable that a detailed study of the respiratory regulation in the crayfish be made in that the respiratory system, morphologically at least, resembles that of the lobster (*Homarus americanus*) very closely. The close resemblance of the two Crustaceans in this respect would suggest that respiratory regulation in

the two species would also closely approximate one another. The widely divergent environments, however, of the two species suggested the finding of a possible difference in degree of respiratory regulation. The results to be described indicate, in general, comparative independence of the oxygen tension present, coupled with an unusual degree of variation in oxygen consumption during consecutive hours when maintained under the same tensions. This latter fact has made inadvisable the grouping together and treatment of the data by statistical methods. Consideration of the animals as individuals, however, reveals certain distinct trends and facts which seem valuable in adding information toward the solution of the problems and generalizations as previously stated.

MATERIALS AND METHODS

The species of crayfish used for all tests made was that of *Cambarus immunis* (Hagen). They were collected as needed from a small pond near Iowa City, Iowa. When brought to the laboratory, the more healthy individuals were selected and placed in a balanced aquarium resembling as nearly as possible their natural environment. The animals were collected, in general, not more than four days before being tested. All collections were made during June and July.

The respiration of all individuals tested was based on their oxygen consumption per hour. In determining the latter, Birge and Juday's (1911) modification of the Winkler method was employed. The animals were in general tested singly. For this purpose rubber-stoppered $\frac{1}{2}$ liter, 1 liter, and 2 liter Erlenmeyer flasks provided with suitable inlet and outlet tubes were employed. The size of the animal determined the size of flask used. It was essential in this regard to place the animal in sufficient water so that the oxygen tension of the latter would not be lowered too rapidly. In this way the oxygen consumption over a wide range of tensions could be studied.

The tests were usually started at 8:00 A.M., and oxygen consumption determinations were made at the end of each hour thereafter, until the animals became asphyxiated or died. For the titrations, 30 cc. samples were drawn each hour. In no case did this constitute more than 6 per cent of the total quantity of water present

in the flasks. In all cases the water drawn was replaced by water of approximately the same tension. The oxygen content of the water added was determined for each addition, and the resulting total amount of oxygen present at the beginning of each hour could therefore be calculated. Some difficulty was experienced at first in securing water of low oxygen tension for addition to the flasks when the oxygen tension of the water in the latter dropped below 30 per cent saturation. In this connection it may be stated, parenthetically, that the crayfish under experiment were able to live under very low oxygen tensions and that the addition of but 30 cc. of slightly lowered oxygen tension water per hour contained enough oxygen to keep the animal alive for days. It was therefore necessary to add water of very low oxygen tension during these periods of the test. It was finally found that such water could be easily obtained by confining a dozen or more crayfish in a jar of water until they were asphyxiated. This water could then be diluted to any desired oxygen tension for addition to the flasks. Low-oxygen-tension water thus obtained seemed to have decided advantages over water deoxygenated by chemical means. The method was also more efficient than that of boiling the water.

In the group testing, ten crayfish were placed in a 5 gallon jar of water. The jars were fitted with outlet tubes and a glass stirring device for the thorough mixing of the water before samples were drawn. Because of the large initial quantity of oxygen present in these tests, it was not necessary to add water at the conclusion of the hourly tests, since the oxygen tension in the jars dropped very slowly. The decrease in the total oxygen available for the animals was determined for each hour, however, in order that the amount consumed per hour could be more accurately determined. The surface of the water in the jars was fully protected against diffusion of atmospheric oxygen by a 2 inch layer of petrolatum. Both the outlet tube and the stirring device passed through the petrolatum. As the animals became asphyxiated or died under low oxygen tensions, they were removed by means of a glass hook. This was found necessary since, as will be described later, asphyxiated and especially dead crayfish have a tendency to add oxygen to the water instead of removing it. Preliminary tests showed that the diffusion of atmos-

pheric oxygen through the petrolatum layer was negligible if not entirely inhibited.

Distilled water was employed throughout in order to obviate the possible ionic effect of chemicals normally present in tap water. In general the water used was approximately 100 per cent saturated at 25° C. partial pressure. The pH of such water averaged 6.7. All animals were starved for 48 hours before testing, and the testing flasks were immersed in water maintained at 25° C. No attempt was made to inhibit the activity of animals during the time they were confined to the flasks, since it was desirable to watch the effect of low oxygen tensions on such possible activity. As a matter of fact, however, the crayfish under experiment remained quiescent over long periods of time, sometimes for 5 or 6 hours without changing their position. Not infrequently the animal would move about for a few seconds. This seemed to become more prevalent as the oxygen tension was lowered. In a few cases the activity became excessive, and it was necessary to discard the data from such individuals. In general, activity was at a minimum and could not have seriously affected the oxygen consumption.

The animals were weighed to the tenth of a gram following the testing period, although this was not essential except as an indication of their size. No attempt was made to calculate the oxygen consumption per gram weight, since the tests were designed to determine the effect on the respiration of the animal as a whole under different oxygen tensions, the comparison of the metabolic rates between individuals of different weights and ages not being under consideration.

The samples of water drawn from the flasks were immediately titrated to avoid possible errors due to standing. N/300 thiosulphate was employed in place of the usual N/100 in order to lend greater accuracy to the tests. A Schellbach burette graduated to the 1/100 cc. was used in titrating, all titrations, whether by day or night, being read under a constant diffused light.

NORMAL VARIATION IN OXYGEN CONSUMPTION

Preliminary tests had shown that considerable variation existed in the oxygen consumption of animals when maintained under a

constant oxygen tension. It was thought advisable, therefore, to make a thorough study of such variation, in that such a factor must be considered in determining the possible regulation of respiration of animals during various oxygen tensions. Thirty individuals were therefore selected, ranging in weight from 3.0 grams to 28.2 grams, and their oxygen consumption determined, individually, for 5 consecutive hours. These tests were made in distilled water and under experimental conditions as previously described. The oxygen tension of the water at the beginning of the tests varied between 88 per cent and 116 per cent saturation at 25° C. partial pressure. For any one animal, however, the difference in the per cent saturation of the water between any two of the five consecutive tests did not exceed 3 per cent, which is a negligible amount considering the wide range of oxygen tensions investigated in connection with the main series to be discussed later. For the majority of animals, the variation between any two tests was but 1 or 2 per cent saturation. In the actual testing of the animals, the experimental flasks were emptied at the end of each hour and fresh distilled water from the same stock added for the next test. In this way the oxygen tension and purity of the water at the beginning of each hour was maintained quite uniform.

The data covering this series are represented in Table I. It will be noted that, in general, the variation in oxygen consumption is considerable between consecutive hours, although in a few cases (for example, animals 20, 28, and 30) the variation is quite small. Averages computed for the total amount of oxygen consumed by the entire group of thirty animals during each of the five consecutive hours reveal, perhaps, a better estimate of the variation present. The fifth-hour average in this regard is approximately 21 per cent lower than the first-hour average, although but 7 per cent lower than the third-hour average, the next lowest as compared with that of the first hour. It should be borne in mind, however, that the hour-by-hour variation of most individuals exceeded this per cent variation.

For the majority of animals tested (see Table I) the amount of oxygen consumed during the first hour was greater than that consumed during any of the following four hours. This was no doubt due to the fact that during the first-hour tests the animals were

placed in water at 25° C., a somewhat higher temperature than that of the stock aquaria in which the animals had been confined prior to testing. The oxygen consumption of ten crayfish, each tested in

TABLE I

NORMAL VARIATION IN OXYGEN CONSUMPTION OF CRAYFISH

ANIMAL	SEX	WEIGHT IN GRAMS	CENTIMETERS OF OXYGEN CONSUMED DURING CONSECUTIVE HOURS				
			First	Second	Third	Fourth	Fifth
1.....	♀	3.0	.755	.628	.510	.606	.507
2.....	♀	3.7	.547	.827	.758	.623	.804
3.....	♂	4.0	.497	.349	.448	.494	.383
4.....	♂	4.3	.788	.472	.434	.839	.632
5.....	♂	4.4	.763	1.059	.541	.516	.327
6.....	♀	4.7	.785	.414	.489	.779	.618
7.....	♂	4.8	.903	.504	.444	.891	.598
8.....	♀	4.8	.582	.346	.315	.425	.366
9.....	♂	5.2	1.402	.963	.878	.944	.668
10.....	♂	5.2	1.523	1.607	1.335	1.148	1.088
11.....	♀	5.3	1.162	1.068	1.027	.819	.959
12.....	♀	5.4	1.442	1.072	.827	.891	.895
13.....	♀	5.6	1.127	.812	.905	.748	.564
14.....	♀	5.9	1.015	.529	.365	.421	.588
15.....	♀	6.2	.954	.813	.773	.658	no test
16.....	♂	6.3	1.295	1.538	1.724	1.147	1.920
17.....	♂	6.5	1.227	.997	1.036	.843	.805
18.....	♀	6.5	1.202	.822	.692	.885	.822
19.....	♀	7.6	1.419	1.104	1.270	.871	.964
20.....	♀	7.8	1.863	1.948	1.990	1.875	1.924
21.....	♂	7.8	1.343	1.463	1.715	1.495	1.728
22.....	♂	9.4	1.721	1.075	1.530	1.382	no test
23.....	♂	9.5	1.629	1.234	1.862	1.310	no test
24.....	♀	13.3	2.326	1.510	1.273	1.295	1.524
25.....	♂	15.5	3.066	2.511	2.799	2.683	2.548
26.....	♂	15.5	1.774	1.627	1.504	1.519	1.233
27.....	♂	20.0	5.422	4.345	4.146	4.457	3.272
28.....	♂	20.0	1.991	2.097	2.044	1.932	2.224
29.....	♂	24.6	5.484	4.725	4.625	4.131	3.328
30.....	♂	28.2	4.790	4.591	4.845	4.814	4.614
Average..	.....	.....	1.693	1.495	1.437	1.381	1.330

water at aquarium temperature and at 25° C., confirm this conclusion. During the first hour, this rise in temperature, therefore, was responsible for a slight raising of the metabolic rate, which appears to have been only a temporary condition, since a common level, in general, was maintained during the following four testing periods.

There is no evidence to show that young crayfish exhibit a greater

amount of variation as compared with older animals, or vice versa. There is also no indication of a sexual difference regarding either the degree of variation present or the amount of oxygen consumed.

OXYGEN CONSUMPTION UNDER DIFFERENT OXYGEN TENSIONS

The complete data for all animals tested under various oxygen tensions is represented in Tables II–VI inclusive. The animals, as previously described, were tested in distilled water at 25° C. This

TABLE II

OXYGEN CONSUMPTION OF CRAYFISH AT VARIOUS OXYGEN TENSIONS

Hours Tested	Animal 1 (1.5 Gm. ♀)		Animal 2 (1.7 Gm. ♀)		Animal 3 (3.0 Gm. ♀)		Animal 4 (4.3 Gm. ♂)		Animal 5 (4.5 Gm. ♀)	
	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed
0	85	.....	85	.....	85	.....	85	.....	85	.....
1	79	.276	78	.208	73	.362	67	.598	61	.738
2	70	.097	67	.188	53	.425	43	.531	40	.521
3	59	.112	54	.221	36	.385	24	.497	22	.478
4	58	.107	46	.148	21	.367	10	.283	12	.270
5	41	.334	42	.032	13	.202	10	.129	10	.062
6	34	.141	29	.303	10	.094	8	.037	8	.035
7	28	.098	23	.078	9	.005	9	.015†	9	.013†
8	21	.170	19	.148	8	.046	8	.029	8	.031
9	19	.032	15	.092	8	.004	8	.007	8	.016
10	15	.096	13	.025	8	.016	8	.002	8	.004
11	13	.031‡	12	.025‡	7	.003‡	7	.027‡	8	.012‡

* The per cent saturation for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The per cent saturation for all hours tested represents the oxygen tension of the water at the end of the hour.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of the animal during the hour.

water varied for different individuals between 85 and 132 per cent saturation, the exact per cent saturation used for each animal being indicated in the tables. Because of the fact that the animals reduced the oxygen tension of the water each hour to a different level by their respiration, it is difficult to group the data so that even four or five tests fall on the same oxygen tension. Furthermore, in cases where this occurred, the same four or five animals did not lower the oxygen tension during the following hours to exactly the same level. Consequently, it is necessary to consider (1) the individual behavior of

the animals undergoing successive hourly tests, and (2) the behavior of groups of individuals arranged according to their weights.

1. INDIVIDUAL BEHAVIOR UNDER VARIOUS OXYGEN TENSIONS

In general the oxygen consumption for the first hour was higher than that obtained for any of the succeeding hours, regardless of the oxygen tension of the water with which the test was started.

TABLE III

OXYGEN CONSUMPTION OF CRAYFISH AT VARIOUS OXYGEN TENSIONS

Hours Tested	Animal									
	6 (6.3 Gm. ♀)		7 (6.5 Gm. ♂)		8 (7.5 Gm. ♂)		9 (7.9 Gm. ♀)		10 (8.1 Gm. ♂)	
	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed
0	106	.....	106	.....	132	.....	132	.....	112	.....
1	94	.767	88	1.146	114	1.123	115	1.062	97	1.747
2	84	.565	76	.660	91	1.279	103	.602	80	1.984
3	75	.483	64	.753	68	1.344	87	.807	69	1.275
4	59	.968	56	.439	43	1.478	66	1.229	54	1.755
5	51	.515	48	.519	29	1.104	56	.533	45	1.251
6	41	.605	40	.540	21	.565	47	.608	35	1.394
7	33	.499	32	.589	22	.013	34	.802	27	1.100
8	.....	.....	.....	.....	20	.165	29	.301	19	1.015
9	.....	.....	.....	.....	17	.117‡	20	.599	15	.825
10	.....	.....	.....	.....	.....	.....	16	.293	19	.198†
11	.....	.....	.....	.....	.....	.....	19	.187†	15	.773
12	.....	.....	.....	.....	.....	.....	14	.377	9	.787
13	.....	.....	.....	.....	.....	.....	14	.011†	8	.273
14	.....	.....	.....	.....	.....	.....	20	.316†‡	8	.068
22	11	.415§	16	1.084§	.....	.....	.....	.....	.....	.....
23	.....	.....	.....	.....	.....	.....	.....	.....	13	.273†§

* The per cent saturation for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The per cent saturation for all hours tested represents the oxygen tension of the water at the end of the hour.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of the animal during the hour.

§ Indicates that death occurred at some time since the last recorded test. The oxygen consumed is also for that period of time.

This fact can be attributed, as in the case of the results obtained for the normal variation in oxygen consumption, to the temperature of the water used in the experiments, as compared with the relatively cooler water of the stock aquaria. Following the first-hour test the oxygen consumption for successive hours underwent marked variation. In some cases relatively high and low amounts were consumed

on alternate hours. In others a steady decline in oxygen consumption occurred, only to be followed by a rise which sometimes became as high as the first few hours. In short, the variation in oxygen consumption during consecutive hours was of the same order as shown for the normal variation in oxygen consumption of animals tested

TABLE IV

OXYGEN CONSUMPTION OF CRAYFISH AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	11 (8.6 Gm. ♀)		12 (9.2 Gm. ♀)		13 (9.5 Gm. ♂)		14 (10.2 Gm. ♀)		15 (10.4 Gm. ♀)	
	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.*	Cc. O_2 Consumed
0.....	100	.....	106	.....	132	.....	113	.....	106	.....
1.....	88	1.315	90	1.785	116	1.049	95	2.026	83	1.459
2.....	82	.800	74	1.938	103	.583	70	2.701	56	1.580
3.....	72	1.144	58	1.931	88	.820	47	2.471	30	1.573
4.....	61	1.443	46	1.540	76	.630	30	1.896	18	.745
5.....	52	1.163	33	1.682	60	1.161	19	1.157	12	.356
6.....	43	1.169	22	1.392	48	.595	14	.638	11	.087
7.....	43	.181	16	.842	34	.802	13	.138	12	.015†
8.....	32	1.660	12	.609	26	.476	14	.059†	11	.119
9.....	26	.717	12	.189	25	.132	12	.212	10	.100
10.....	24	.458	13	.054‡	21	.298	.....	.....	10	.022
11.....	14	1.462	.....	.....	20	.074	.....	.....	.....	.....
12.....	16	.050	.....	.....	20	.006	.....	.....	.....	.....
13.....	19	.112†	.....	.....	16	.271	.....	.....	.....	.....
14.....	15	.745	.....	.....	.....	.....	.....	.....	.....	.....
24.....	19	.170†§	.....	.....	.....	.....	10	.278§	.....	.....
29.....	.....	.....	.....	.....	26	.651†§	.....	.....	.....	.....

* The per cent saturation for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The per cent saturation for all hours tested represents the oxygen tension of the water at the end of the hour.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of the animal during the hour.

§ Indicates that death occurred at some time since the last recorded test. The oxygen consumed is also for that period of time.

hourly in water of constant oxygen tension. This was true, in spite of the fact that the oxygen tension of the water in the experimental flasks steadily dropped as the animals consumed more and more oxygen. Eventually, however, there came a time when such regulation of respiration irrespective of oxygen tension ceased. The oxygen tension at which such regulation ceased was subject to variation depending on the animal. For a few, regulation ceased when the tension was lowered to approximately 35 per cent saturation. At the

lower extreme, regulation was maintained down to about 15 per cent saturation. The majority, however, were able to maintain their normal respiration down to between 25 and 19 per cent saturation. If the data for all the tests of the entire twenty animals tested be grouped together and the average oxygen consumption calculated

TABLE V

OXYGEN CONSUMPTION OF CRAYFISH AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	16 (12.0 Gm. ♀)		17 (14.9 Gm. ♂)		18 (16.4 Gm. ♀)		19 (18.5 Gm. ♀)		20 (18.7 Gm. ♀)	
	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed
0	117	.....	122	.....	122	.....	127	.....	127	.....
1	99	2.111	92	3.405	82	4.598	92	4.013	100	3.185
2	82	1.241	56	4.015	50	3.678	63	3.167	71	3.207
3	73	1.394	33	2.504	25	2.764	34	3.290	43	3.131
4	58	1.529	22	1.197	17	.895	21	1.439	24	2.256
5	55	.271	17	.654	13	.461	14	.841	16	.827
6	38	1.883	14	.286	11	.234	13	.062	13	.413
7	30	.982	15	.122†‡	13	.142†‡	12	.159	14	.109†
8	21	.887	.....	.....	.....	.....	11	.099	9	.587‡
9	20	.120	.....	.....	.....	.....	10	.158	.....	.....
10	19	.190	.....	.....	.....	.....	8	.190	.....	.....
11	.....	.....	.....	.....	.....	.....	9	.047†	.....	.....
12	.....	.....	.....	.....	.....	.....	10	.126†	.....	.....
13	.....	.....	.....	.....	.....	.....	11	.043†	.....	.....
22	9	1.199§	.....	.....	.....	.....	.....	.....	.....	.....
23	.....	.....	.....	.....	.....	.....	11	.029†§	.....	.....

* The per cent saturation for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The per cent saturation for all hours tested represents the oxygen tension of the water at the end of the hour.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of the animal during the hour.

§ Indicates that death occurred at some time since the last recorded test. The oxygen consumed is also for that period of time.

for each 10 per cent drop in oxygen tension, the average respiratory regulation appears to be in the neighborhood of 30 per cent saturation.

An interesting correlation may be drawn between the age of the animal, as indicated by his weight, and the range of respiratory regulation possessed. Animals 1, 2, 3, 4, 5, 8, and 9, ranging in weight from 1.5 grams to 7.9 grams and with an average weight of 4.3 grams, show regulation down to about 20 per cent saturation.

Animals 6, 7, 10, 11, 12, 13, 14, 15, and 16, ranging in weight from 6.3 grams to 12.0 grams and with an average weight of 9.0 grams, show regulation down to about 30 per cent saturation. Animals 17, 18, 19, and 20, ranging in weight from 14.9 grams to 18.7 grams and with an average weight of 17.1 grams, show regulation down to approximately 40 per cent saturation. This would seem to indicate clearly that the older the animal, the less the range of regulation, and vice versa.

Below the lower limit of the range of respiratory regulation, the oxygen consumption becomes very erratic and the animals are apparently unable to adjust themselves to the lowered oxygen tensions. The animals frequently become more active and swim to the top of the flasks in search of oxygen. Eventually, aphyxiation sets in, which process may be rapid or long drawn-out, depending on the individual. For most individuals, however, the first signs of asphyxiation are discernible between the tensions of 15 per cent and 10 per cent saturation. The steps in asphyxiation are quite definite regardless of the size or sex of the animal. A general lack of vigor is first noted, the animal appearing to move about with difficulty. In some cases reddish cross-lines are discernible on the dorsal side of the abdomen between the segments, the exact nature of which was not determined. When movement finally ceases, the animals have just enough strength left to right themselves when turned on their backs. When this power is lost, they still continue to move their appendages feebly and the respiratory movements of the mouth parts cease entirely. In this condition they may lie for hours, consuming little or no oxygen. The last stage consists of the cessation of all movement, the animal appearing lifeless. If, however, the individual is placed in fresh water of high oxygen content, recovery occurs in the majority of cases. Death can generally be recognized by the curling up of the posterior segments of the abdomen. Complete asphyxiation usually occurred in water of 8–10 per cent saturation.

An interesting phenomenon occurred in most cases between the saturations of 20 per cent and 8 per cent. The animal appeared practically to cease respiration for an hour or more, and the tests for such periods indicated an addition of oxygen to the water, presumably by

the animal. This reversal of oxygen consumption was in general followed by respiration considerably higher in amount than experienced during the hours immediately preceding the cessation of the respiration period. Inhibition of respiration sometimes occurred again during the succeeding hours tested. Following the death of the animal, the body generally liberated oxygen enough to raise the oxygen tension of the water 1 per cent or 2 per cent. The possible explanation of this phenomenon will be discussed later.

2. GROUP TESTING UNDER VARIOUS OXYGEN TENSIONS

The testing of animals as regards their respiratory regulation in groups of ten was thought advisable in that it would serve to indicate directly the average behavior of the individuals concerned. The data for the three groups tested are represented in Table VI. The equipment used and method of group testing have already been described.

Group 1 consisted of ten crayfish ranging in weight from 16.0 grams to 19.2 grams and with an average weight of 17.3 grams. Group 2 consisted of ten crayfish ranging in weight from 11.5 grams to 23.6 grams and with an average weight of 18.0 grams; while the ten animals of Group 3, ranging in weight from 10.5 grams to 19.1 grams, had an average weight of 15.6 grams.

The general behavior and oxygen consumption of all three groups resembled, in general, the conditions found true for the individual testing of animals as previously described. The oxygen consumption with its corresponding drop in oxygen tension of the water was especially great during the first hour. During the next two or three hours the oxygen consumption varied according to the normal variation observed for most of the crayfish tested. The respiratory regulation for the three groups appeared normal down to approximately 40 per cent saturation. The average weight of the entire thirty animals tested amounted to 17.0 grams. Compared with animals 17, 18, 19, and 20, whose average weight was 17.1 grams, the range of respiratory regulation will be seen to be practically the same.

Respiration below 40 per cent saturation was likewise erratic, as previously described for the individual animal tests. The peculiar phenomenon of oxygen liberation by the animals during the lower oxygen tensions was especially prevalent. Since the crayfish were

removed from the testing jars as soon as complete asphyxiation occurred, the oxygen consumption for the following hours must be considered as due to the remaining individuals.

TABLE VI

COMBINED OXYGEN CONSUMPTION OF TEN CRAYFISH AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	GROUP 1 (Av. Wt. 17.3 Gms. Ten ♀)		GROUP 2 (Av. Wt. 18.0 Gms. Ten ♀)		GROUP 3 (Av. Wt. 15.6 Gms. Seven ♀ Three ♂)	
	Per Cent Sat.*	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed	Per Cent Sat.	Cc. O_2 Consumed
0	103	.....	102	.....	110	.....
1	76	30.100	69	36.192	77	36.367
2	47	32.172	47	23.860	58	21.094
3	37	10.856	34	14.784	42	17.749
4	21	16.998	22	13.154	26	17.353
5	18	3.760	15	7.121	17	9.328
6	14	3.618	16	.264†	17	.280
7	16	1.222†	13	3.058	13	5.086
8	12	4.144	12	1.115‡	14	.996†‡
9	12	.050	11	.578	11	3.179‡‡
10	12	.050	12	.483†	12	1.524†
11	12	.050	11	.686	11	1.082
12	11	1.307	11	.457	10	.775
13	10	.156‡	10	.585	12	1.713†
14	14	3.047†	10	.245	11	1.591†‡
15	15	.432†‡	10	.356‡‡‡	9	1.561‡
16	9	6.486	10	.275†‡	10	.665†
17	10	1.685†	9	.868‡	9	1.436
18	11	.985†‡‡	14	4.595†	10	1.064†
19	10	1.072‡‡‡	9	5.691	10	.147‡‡‡
20	10	.277	9	.166†	8	1.618‡
21	8	1.985	10	1.794†	11	2.647†
22	10	1.421†	9	1.141	10	.439
23	8	1.190	7	1.959	9	1.279‡
24	.....	.....	7	.233	.....	.....
25	.....	.....	5	1.893	.....	.....
26	.....	.....	10	4.114†‡	.....	.....
27	.....	.....	8	1.717‡	.....	.....
28	.....	.....	7	.665	.....	.....
29	.....	.....	9	1.883†‡	.....	.....
30	.....	.....	12	2.724†‡	.....	.....
31	8	.335§	.....	.....	.....	.....
32	8	.507‡	.....	.....	.....	.....

* The per cent saturation for hour 0 represents the oxygen tension of the water in the experimental jar at the beginning of the test. The per cent saturation for all hours tested represents the oxygen tension of the water at the end of the hour.

† Indicates the addition of oxygen to the water by the animals, instead of oxygen being removed.

‡ Each ‡ indicates the asphyxiation or death of an animal during the hour.

§ The § indicates the death of two animals at some time since the last recorded test. The oxygen consumed is also for that period of time.

The animals undergoing testing will be seen by reference to Table VI to exhibit widely varying degrees of resistance to asphyxiation. A few became asphyxiated when the oxygen tension dropped to from 14 per cent to 11 per cent saturation. The majority, however, were able to adapt themselves to the lower tensions so that asphyxiation did not occur until 5 to 8 hours later. Several remained in a weakened condition in various stages of asphyxiation for prolonged periods of time. In Group 2, for example, four animals resisted complete asphyxiation until the sixteenth, seventeenth, nineteenth, and twentieth hour, respectively, after the oxygen tension of the water had dropped to 12 per cent saturation. Three animals of Group 1 resisted asphyxiation until the twenty-third and twenty-fourth hours after the oxygen tension had reached 12 per cent saturation. In this latter group it was necessary to run the test for thirty-two hours before all animals were completely asphyxiated. The majority of the animals asphyxiated in the group testing recovered when placed in fresh water of high oxygen tension.

EFFECT OF H-ION CONCENTRATION ON OXYGEN CONSUMPTION

The respiration of the crayfish under analysis in the present paper would logically be expected to increase the hydrogen-ion concentration of the water in the experimental flasks to which they were confined. That this increase might prove to be of some magnitude likewise suggested itself as being plausible in view of the prolonged periods of time covered by the tests. Consequently, hydrogen-ion tests were made in connection with representative members of all oxygen consumption tests made. This included individuals of both sexes and of all sizes. Such tests revealed the fact that the greatest increase in hydrogen-ion concentration consisted in a drop in pH from 6.8 to 5.9. All other individuals tested increased the hydrogen-ion concentration to a pH intermediate between these two extremes. The natural inference suggested by this is that the increase in hydrogen-ion concentration might have influenced, in some manner, the oxygen consumption obtained for the individuals being tested. Consequently, an experiment was devised to determine the effect, if any, of hydrogen-ion concentration on the respiration of crayfish.

The technique employed in these tests was, briefly, as follows:

Five crayfish were selected of varying weights (see Table VII) and their oxygen consumption determined individually for six consecutive hours. The six consecutive tests were run in distilled water showing a pH at the beginning of the tests of 6.8, 6.0, 5.2, 6.7, 6.1, and 5.2, respectively. These different hydrogen-ion concentrations were obtained, artificially, by the diffusion of CO_2 through the water. The oxygen tension of the water was also the same at the beginning of each hour. The range of pH thus investigated was more than that exhibited by any of the animals of the oxygen-tension series. Moreover, the change of pH was far more rapid than in the latter series and involved a sudden raising of the pH between the third

TABLE VII

EFFECT OF H-ION CONCENTRATION ON OXYGEN CONSUMPTION OF CRAYFISH

ANIMAL	SEX	WT. IN GMS.	CONSECUTIVE HOURS											
			First		Second		Third		Fourth		Fifth		Sixth	
			pH	Cc. O_2 Consumed	pH	Cc. O_2 Consumed	pH	Cc. O_2 Consumed	pH	Cc. O_2 Consumed	pH	Cc. O_2 Consumed	pH	Cc. O_2 Consumed
1.....	♀	3.0	6.8	.354	6.0	.333	5.2	.416	6.7	.238	6.1	.330	5.2	.284
2.....	♀	5.1	6.8	1.179	6.0	1.130	5.2	1.190	6.7	.884	6.1	.841	5.2	.858
3.....	♀	8.2	6.8	1.421	6.0	1.307	5.2	1.624	6.7	1.262	6.1	1.321	5.2	1.207
4.....	♀	15.1	6.8	1.594	6.0	1.751	5.2	1.874	6.7	1.475	6.1	1.297	5.2	1.249
5.....	♀	18.2	6.8	2.113	6.0	1.359	5.2	2.071	6.7	1.914	6.1	2.026	5.2	1.836

and fourth hours which condition would tend to bring out any possible effect of hydrogen-ion concentration on oxygen consumption. This raising of the pH, however, never occurred in the oxygen-tension series, since the pH dropped steadily from the first hour of the tests to the last.

The data for the hydrogen-ion concentration tests are represented in Table VII. Reference to the oxygen consumed during each consecutive hour will reveal the fact that no marked effect was produced in any of the five animals tested. It is true that variation in oxygen consumption occurred, but such variations cannot be correlated with the change in pH or CO_2 content at which the tests were run. Furthermore, the variation present resembled that previously described and represented in Table I for the normal variation in oxygen consumption of animals tested for consecutive hours at ap-

proximately the same oxygen tension and pH. The conclusion to be drawn, therefore, is that within the range of pH and CO_2 content tested, changes in hydrogen-ion concentration and CO_2 content have no marked effect on the oxygen consumption of crayfish.

DISCUSSION

The normal variation in oxygen consumption is of such a magnitude in certain cases that oxygen consumption must be viewed as an unreliable measure of the metabolic rate for any particular hour. Fluctuations in the metabolic rate of invertebrates of such pronounced proportions are unknown to the writer. It seems more likely that some system whereby a reserve oxygen supply is maintained exists in the crayfish. When this reserve reaches a certain low point, as it is used up by the various tissues of the body, the external respiration of the animal then increases to build the reserve up to maximum. In this way a steady supply of oxygen would be distributed to the various tissues. It was noticed in a large number of cases that high oxygen consumption was correlated with increased activity of the mouth parts. Although the movement of the scaphognathites was maintained fairly constant from hour to hour, the movements of the mouth parts would govern to some extent the flow of water through the gill chambers.

A more logical and simpler explanation might be that the individual cells of the animal are capable, in themselves, of storing surplus oxygen which is then utilized at a certain rate in connection with their various metabolic activities until the supply is depleted. The reserve supply could then be thought of as being re-established. In this way the oxygen tension of the blood would be subject to some variation, and the attempt on the part of the animal to maintain it at a constant level would result in a variation of the amount of oxygen consumed. It is interesting to mention in this connection the peculiar power of crayfish to live for some time when removed from the water. Whether or not atmospheric oxygen is utilized at this time or oxygen is derived from an internal reserve supply has not been determined. The problem is an interesting one, however, and warrants investigation.

The data covering the range of respiratory regulation of indi-

viduals under different oxygen tensions indicate quite clearly that crayfish exhibit respiratory regulation over a considerable range. This is in direct contrast to Amberson, Mayerson, and Scott's (1924–25) work on the lobster, where oxygen consumption was found to be proportional to the oxygen tension of the sea water. The contrast is interesting in that both organisms possess practically the same respiratory system, structurally. Such a contrast seems plausible to the writer in view of the wide differences in physiological properties between sea water and distilled water. Furthermore, there is no logical reason why two structurally similar respiratory systems should function similarly in all respects.

The erratic respiration of crayfish below the lower limit of respiratory regulation suggests, at least, that physiological regulation of respiration can no longer be maintained in a normal manner and hence is followed shortly by disturbances leading to the asphyxiation of the animal. That certain individuals are able to resist the onset of asphyxiation for some time appears evident from the data presented. Evidently they possess a greater degree of adaptability in this regard. This would seem especially applicable to the species of crayfish investigated, in that crayfish living in ponds are normally subject to considerable variations in the oxygen tension of the water, the latter being due to temperature changes and reduced volumes during droughts. The writer has kept one crayfish in the laboratory for over three months in a small jar of water whose oxygen tension averaged 26 per cent saturation at room temperature. It seems possible, therefore, that pond and sluggish-water crayfish possess a certain degree of adaptability to lowered oxygen tensions. Whether or not this adaptability is shared by crayfish living in swift-running streams has not been determined. The inference, however, is that such forms, not being normally subjected to great variations in oxygen tension, would not possess so great a degree of adaptability in this respect.

The fact that older crayfish appear unable to regulate respiration under as low an oxygen tension as young animals presents a situation difficult of explanation. Certainly, the completeness of the structural development of the respiratory system cannot be used here as an explanation, since the older animals must possess a

respiratory system as fully developed as, if not more fully developed than, that of the young animals. It can only be suggested here that the body cells of older animals may have lost in some way the physiological adaptability of the body cells possessed by young animals toward low oxygen tensions.

The phenomenon presented by many of the tested animals whereby an apparent reversal of oxygen consumption occurs when under low oxygen tensions is likewise difficult of adequate explanation. The fact that such liberation of oxygen by the animal took place only during comparatively low oxygen tensions would again suggest the possibility of a temporary loss in the power of the body cells to retain a reserve supply of oxygen in a normal manner. It seems possible that under lowered tensions the oxygen reserve may be suddenly released. The fact that during the following hour or hours oxygen was in general consumed again by the animals indicates a partial recovery of oxygen retention. In general, it will be noted, however, that such reversals in oxygen consumption became more frequent after having first occurred, and were especially prevalent following the onset of asphyxiation.

That changes in the hydrogen-ion concentration and CO_2 content of the water may have influenced the rate of oxygen consumption was shown to be without foundation in view of the tests made concerning this point. For all practical purposes, the variation in oxygen consumption during changing hydrogen-ion concentrations and CO_2 content might have been employed in representing the normal variations in oxygen consumption of animals in water of a constant pH and CO_2 content. This finding coincides with Amberson, Mayerson, and Scott's (1924–25) results on the lobster, where changes in the CO_2 tension of the sea water, much exceeding a range of pH 8.1–7.6, were found to have no effect on the rate of oxygen consumption.

During the course of the work described in the present paper, eight cases of molting were observed which occurred during the testing periods. Although data from such animals were discarded, it was noted that no marked changes in oxygen consumption occurred which might be attributed to this process. Five of these cases survived the process, possibly because of the fact that molting occurred

when the water in the experimental flasks was still at a relatively high oxygen tension. The remaining three died shortly after or during the process. The oxygen tension of the water at which these three molted was relatively much lower than in the case of the five surviving the molting process, which fact possibly accounted for their death.

SUMMARY AND CONCLUSIONS

1. Thirty crayfish (*Cambarus immunis*) were tested for five consecutive hours using a modified Winkler method for determining oxygen consumption. The hourly variation in oxygen consumption was found, in general, to be considerable, although the tests were made with distilled water at a constant temperature, oxygen tension, and hydrogen-ion concentration. Attention is called to the fact that such variation must be considered in the analysis of oxygen consumption under various oxygen tensions.

2. The oxygen consumption of twenty crayfish was determined, individually, for consecutive hours in water undergoing a decrease in oxygen tension resulting from the metabolism of the animals. Regulation of respiration was found to be present down to 20 per cent saturation for animals averaging 4.3 grams in weight, down to 30 per cent saturation for animals averaging 9.0 grams in weight, and down to 40 per cent saturation for animals averaging 17.1 grams in weight. The conclusion is drawn that the older the animal, the less the range of respiratory regulation.

3. Below the lower limit of the range of respiratory regulation, oxygen consumption becomes erratic. Cases are cited where an apparent reversal in oxygen consumption occurs, the animals liberating oxygen to the water at low oxygen tensions. Asphyxiation occurs at low tensions in definite stages and may be rapid or considerably extended.

4. Thirty crayfish were tested in groups of ten each to determine the effect on oxygen consumption under decreasing oxygen tensions. The results were similar to those obtained with crayfish tested individually having the same weight.

5. Five animals were tested for six consecutive hours in water having hydrogen-ion concentrations of pH 6.8, 6.0, 5.2, 6.7, 6.1, and 5.2, respectively. No marked effect on the oxygen consumption due

to changes in hydrogen-ion concentration and CO_2 content were recorded. The conclusion is drawn that within the pH range investigated, hydrogen-ion concentration has no effect on oxygen consumption.

6. Suggestions and possible explanations are offered to explain the normal variation in oxygen consumption, the decrease in range of respiratory regulation for older animals, and the apparent paradox of reversal of oxygen consumption during low oxygen tensions and asphyxiation.

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EFFECTS OF SPERM INJECTIONS INTO FEMALE RABBITS

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THAT the injection of spermatozoa and testicular extracts into an animal may incite the production of spermatotoxins has been the observation of numerous investigators. The earlier studies in this general field were made largely by means of *in vitro* experiments, and the spermatozoa of one species of animal were used as the antigen in the other. Subsequent studies have brought forth reports of isospermatotoxins and the development of shorter or longer periods of sterility in both male and female animals following the repeated injection of spermatozoa. A number of workers have presented evidence that following normal coitus there may be a penetration of spermatozoa into the tissues of the female genital tract, where they may act as an antigen, thus inducing the formation of antibodies against spermatozoa.

The present investigation was undertaken to study further some of the events following injectior of spermatozoa into female rabbits.

LITERATURE

The first reports of the production of spermatotoxins were made almost simultaneously in 1899 by Metchnikoff and Landsteiner. Metchnikoff (1899) injected extracts from the rabbit testis and epididymis into guinea pigs and discovered that the serum of the guinea pig acquired the property of rapidly immobilizing the spermatozoa of the rabbit without, however, causing their dissolution. This antiserum was species specific but was also slightly hemolytic, probably because of the difficulty of preparing an antigen free from blood. Anti-rabbit hemolytic serum, however, was not toxic for spermatozoa of the rabbit. Landsteiner (1899), employing the method of Pfeiffer, found that the immobilization of bull spermatozoa in the peritoneum of the guinea pig is hastened by the previous injection of bull spermatozoa. Moxter (1900) injected a sperm emulsion from

the epididymis of the ram into rabbits and noted that the repetition of this process gave the serum of the treated rabbit marked spermicidal qualities *in vivo* for the same kind of spermatozoa. *In vitro* this serum behaved like normal serum. DeLeslie (1901) rendered male albino mice sterile for from 16 to 20 days by the injection of spermatotoxic serum of the guinea pig. Dunbar (1910) found that spermatotoxic serum will hasten the immobilization of spermatozoa in hanging-drop preparations. Taylor (1908) obtained an anti-serum following immunization with whole sperm. However, when he attempted to determine what chemical fraction of the injected spermatic material is responsible for the production of the cytolytic substances, he found only that the "ether soluble fraction of the spermatozoa of the salmon, like protamin and nucleinic acids, has not the power of causing in the inoculated animal the formation of an antibody capable of producing cytolysis of the spermatozoa of the same fish." Dittler (1920) made intravenous injections of fresh ejaculation fluid into female rabbits and found that sterility may be caused by such injections. This treatment did not seem to interfere with the oestrous cycle of the test animals. Dittler's anti-serum was specific, as negative results were obtained when human sperm were used. Tsukahara (1922) found that the injection of testicular extracts of equine and bovine origin into guinea pigs and rabbits is more toxic for males than for females. If injected in sublethal doses into pregnant females, the testicular extracts may cause death and abortion of the young. Dervieux (1921) claims not only a species specificity but also an individual specificity of spermatotoxins. One may thus, according to Dervieux, determine not only that a given quality of sperm is of human origin but also that sperm comes from one individual and not another.

Guyer (1922) draws the following conclusions from an interesting series of experiments.

1. Spermatotoxic sera, prepared by injecting fowls repeatedly with the spermatozoa of rabbits, are toxic *in vitro* for the spermatozoa of both rabbits and guinea pigs.

2. When introduced into the blood stream of male rabbits at intervals for four or five weeks, such serum produced partial or complete sterility. Inactivation of many spermatozoa, reduction in numbers, or even complete disappearance from the semen occurred.

3. Microscopical examination of the testes of a treated male showed that not only were the mature spermatozoa affected, but disintegrative changes had taken place, or were in progress in the seminiferous tubules.

4. The blood serum of a rabbit injected intravenously with its own spermatozoa becomes highly toxic for the spermatozoa of rabbits, including its own.

5. The spermatozoa of a rabbit which has been repeatedly injected with its own semen are much less viable, both in normal serum and in spermatotoxic serum, than are normal spermatozoa. Presumably such spermatozoa have been influenced specifically *in vivo* by the spermatotoxic serum of their own host.

Oslund (1926) observed degeneration of the germinal epithelium in the testes of some rabbits which had received sperm injections, but cautiously states that "degeneration of germinal epithelium is not necessarily a consequence of such injections of spermatozoa."

McCartney (1923) found that female rats may be sterilized for a period of 2–22 weeks by the injection of spermatozoa or testicular extracts and that the injection of other organ extracts does not cause sterility. This sterility seems to be due to spermatotoxins in the female genital tract, as the sexual cycle of the female rat is not affected by immunization with spermatozoa. Hektoen and Manly (1923) found that the injection of human semen, human seminal fluid, and extracts of human spermatozoa into rabbits induced the formation of precipitins that are specific for human seminal proteins; and that swine, bovine, and equine seminal fluids, injected into rabbits also lead to the production of species- and semen-specific precipitins. In a later paper McCartney (1923) reports that female rats that have been sterilized by subcutaneous injections of rat spermatozoa, on later becoming fertile, cast smaller litters than normal rats. Evidence is also presented that vasoligation in the male rat may be followed by the development of antibodies for semen in the blood. This fact seems to prove the presumption of Guyer (1922) that "the degenerative changes in the seminal epithelium which follow resection or occlusion of the ductus deferens may similarly be due to the action of a spermatotoxin." Kennedy (1924) found that both male and female guinea pigs may be sterilized by the repeated injection of guinea-pig spermatozoa and that the antibody produced in the male is more active than that produced in the female. Kennedy found that if an animal's own spermatozoa were used a more pronounced antibody reaction could be obtained.

Kohlbrugge (1912) has shown that there may be a penetration through the germinal epithelium and an invasion into the underlying connective tissue of the female rat and other rodents by spermatozoa following normal coitus. Waldstein and Ekler (1913) were able to demonstrate post-copulatory ferments which were absent before copulation that led to the absorption of sperm. These workers got a specific Abderhalden reaction in female rabbits for testicular proteins within 24 hours following copulation. This reaction is comparable to that claimed by some to be given by the blood of a pregnant woman to placental material.

Mayer (1922) reports that sexual intemperance in women may lead to the premature rupture of follicles and cause sterility due to abortion of these follicles. Mayer further states that many cases of pregnancy have resulted from periods of sexual abstinence during the late war, the abstinence remedying the condition of sterility due to the overloading of the female genital tract with sperm. Vogt (1922) raises the question if it is not possible for human females to become sterile following too frequent sexual indulgences. He states that often couples who lead normal sexual lives are sterile but that after periods of separation or abstinence, the woman readily becomes pregnant. The fact that among animals the first copulation is often followed by pregnancy and later ones not may thus find explanation in the thesis that the first copulation is the most favorable one, since there is an absence of spermatotoxins which may develop subsequent to the intromission of sperm into the female.

MATERIAL AND EXPERIMENTAL METHODS USED IN THIS STUDY

Because of their prolific breeding habits under the conditions studied, rabbits were used as the experimental animals in this investigation.

Only female rabbits which had previously proven their fertility were used for the production of antisera. Records were kept of all matings and of the number of young born per litter to determine if the injections had any effect on the number of young born during the course of the treatment or upon the return of fertility after a period of sterility. Note was also made of all cases of abortion that

came within observation. With the exception of one experimental series in which material from the epididymis of the albino rat was used, the fresh ejaculatory fluid and extracts of the testes and epididymes of rabbits were used as antigen. To secure sufficient quantities of semen, one or several male rabbits were permitted to copulate a number of times with a female rabbit, and the semen was drawn from the vagina by means of a special pipette described by Guyer (1922).

METHOD OF ANTIGEN PREPARATION

The following procedure was carried out for the preparation of the antigen from the testis and epididymis: The male rabbit or rat was either anesthetized with ether or killed by a blow on the head, and the testis and epididymis removed and washed in sterile physiological salt solution to remove as much of the blood as possible. The epididymis was then removed from the testis and finely sliced and macerated, then shaken vigorously in 10 cc. of sterile Ringer's solution, and then filtered through four thicknesses of sterile cheesecloth. The testis was given the same treatment with the exception that 15 cc. instead of 10 cc. of Ringer's solution was used in the extraction process. Throughout the course of the preparation of the antigen, precautions were taken to keep all of the material intended for injection in an aseptic condition.

Extracts of the salivary glands of some of the same rabbits which furnished the testes and epididymes were likewise prepared in sterile Ringer's solution and used as one of the control antigens.

To rule out the possible antigenic effects exercised by the protein constituents of the seminal vesicle and prostate secretions, the ejaculated material coming from vasectomized rabbits was also employed as control. Two normal male rabbits, whose fertility had previously been established, were rendered sterile by vasectomy. This operation dams back the fluid from the testes. The vasectomized male however retains his copulatory ability. Under these conditions the ejaculate is devoid of spermatozoa but still contains the secretions of the prostate gland and seminal vesicle. Using this sperm-free material for antigen, a number of female rabbits received injections corresponding in amounts and time intervals to those

given to the experimental rabbits. This antigen was collected by means of a vaginal pipette as described above.

INJECTION OF ANTIGEN

Usually the antigen was injected intravenously, although some injections were given intraperitoneally. Aseptic precautions were taken, and in no case was there any observed infection. The injections were usually given in ascending doses in amounts from 0.2 cc. to 4 cc. at intervals of from 3 to 6 days over a period of from 3 to 15 weeks. In the control series similar injections were made of the salivary gland extracts and of the ejaculated material from the vasectomized males. Those rabbits in which it was desired to study whether or not antigenic effects are produced by excessive amounts of spermatozoa in the female genital passages were allowed to copulate as often as one or more males would cover them. Ordinarily, female rabbits will accept the male only during oestrum; but this is by no means always the rule, as it not infrequently happens that they will copulate at various times during their oestrous cycle or even during pregnancy. When it was desired to introduce semen into the genital tracts of females which were not willing to mate, males were allowed to mate with other females, and the ejaculate was collected by means of a pipette and introduced deeply into the vagina of the test animal.

TESTS FOR IMMUNITY

The criteria for the development of spermatotoxins following the injection of semen and extracts from the testis and epididymis were the following:

1. A period of infertility under conditions which, aside from the experimental treatment, normally lead to pregnancy.

2. Destruction of the spermatozoa within the genital passages of the treated female rabbits.

3. Immobilization and agglutination of spermatozoa *in vitro* by the serum and vaginal secretions of the injected rabbits.

If a female rabbit, whose previous history demonstrated her fecundity, later failed to become pregnant as a result of the injections without apparently having her sexual behavior altered in any

way, and she copulated with males as did the control and normal females, it was taken to mean that she became sterile as a result of these injections. At various intervals following copulation during the course of injections, some of the treated female rabbits were killed and their genital tracts removed and opened to study the longevity of the spermatozoa contained therein. Samples of sperm were taken at different levels of the tract by means of a pipette. Under the low power of the microscope it was easy to ascertain whether the spermatozoa were dead or alive.

To test the effect of the serum of the different groups of experimental animals upon spermatozoa, blood was drawn from the marginal ear vein and allowed to clot. One-half cc. of the expressed serum was removed to a depression slide. Using the usual bacteriological loop, a loopful of the sperm suspension from the epididymis similar to that used for injections was mixed with the serum. The slide was then sealed with a cover glass, mounted on a paraffin ring, and placed in an incubator at 26° C. Previous tests showed that *in vitro* this temperature, other factors being constant, is more conducive to the longevity of the sperm than is the temperature of the body. That a medium whose temperature is below that of the animal body is favorable *in vitro* to the life of spermatozoa has been reported by Wolff (1921) and Lewis (1911). Observations were made from time to time to determine the duration of motility and extent of agglutination of the spermatozoa.

The effect of the vaginal secretions, *in vitro*, upon the spermatozoa was studied by inserting cotton swabs into the vaginas of the rabbits and adding a quantity of spermatozoa to a Ringer's solution extract of the material collected on the swabs. The amount of vaginal secretion which can be obtained in this manner is quite variable. Lest different concentrations per se of the Ringer's solution extracts of the vaginal secretions affect the behavior of the spermatozoa, it was decided to use a standard medium. This medium was prepared as follows: A small tuft of cotton was fastened to one end of a slender applicator stick. After being weighed, the swab was inserted into the vagina of the rabbit to a distance of about 5 cm., allowed to remain there about 10 seconds, and then removed. The swab was again weighed, and the increase in weight ascertained. This figure,

in milligrams, was divided by 5, the quotient representing the number of cubic centimeters of Ringer's solution to which the swab was added. Thus, if the swab increased in weight to the extent of 15 mg., because of the absorbed vaginal secretion, it was extracted in 3 cc. of Ringer's solution. The swab was gently agitated in the Ringer's solution and the solution then filtered, ½ cc. of the filtrate being transferred to a depression slide. To this medium was added a loopful of sperm suspension. The slide was then sealed, incubated, and observed by the method described above. The same procedures were carried out in the control experiments.

EXPERIMENT I

SPERMATOTOXINS FROM INJECTIONS OF RABBIT SPERMATOZOA

Eleven normal adult female rabbits received intravenous and intraperitoneal injections of spermatozoa derived from the epididymes of normal adult rabbits. These injections were given in gradually ascending doses and ordinarily at time intervals of from 3 to 6 days. Sometimes a period of from 10 to 20 days or more intervened between the time of the last of a series of injections and the time when injections were again resumed. This time was allowed to elapse in order that a study might be made of the duration of the effects induced by the injection of epididymal material and to determine, if possible, to what extent a lapse of time may modify the degree of response given by spermatozoa in a medium containing the sera or vaginal secretions of the injected rabbits.

Care was taken to duplicate, or at least to simulate, the conditions of experiment in the experimental series with control tests. Ten other normal adult female rabbits were used as experimental controls in this series. Six of these received intravenous injections of Ringer's solution extracts of the salivary glands for rabbits. The 4 remaining control rabbits were given intravenous injections of ejaculate derived from the vaginas of normal rabbits which had mated with vasectomized males.

Aside from the fact that the control rabbits were given injections of other antigens than those used in the experimental series, they received the same treatment as did the latter.

Since one group of rabbits received injections of material coming

from the epididymis (Experiment 1) and another group of rabbits received injections of extracts from the testis (Experiment 2), the injections being given on the same days, the salivary gland and sperm-free ejaculate controls which were used to check the results for Experiment 1 were similarly used as controls for Experiment 2. Only freshly prepared material was used as antigen, and in no case did more than 5 hours intervene between the time of preparation and the time of injection of the antigen.

At various intervals before, during, and following the course of treatment, tests were made to study the viability of rabbit spermatozoa in media containing serum or the vaginal secretions of the experimental and control animals. Each test was accompanied in practically all cases by a control in which the longevity of the spermatozoa, coming from the same source, in the sera and vaginal secretion solutions from normal rabbits was studied. This control would show if any apparent spermatotoxic effect exerted by the sera and vaginal secretions of the treated animals might not, in reality, be attributable to an innate weakened condition of the spermatozoa used in a given test. The experimental controls were designed to show whether the injection of extracts of the salivary gland or a sperm-free ejaculate might incite the production of spermatotoxins. A large number of such tests, many of them in duplicate, were performed. For convenience, the results of some of these tests which are considered to be typical and representative of them all are summarized in Table I.

An examination of Table I shows that the serum and vaginal secretions of female rabbits, which have received repeated injections of rabbit spermatozoa, acquire marked toxic properties for corresponding spermatozoa. Furthermore, the repeated injection of salivary-gland extracts and sperm-free ejaculate does not seem to bring about this result. The criteria for the development of spermacidal substances in the serum and vaginal secretions were the decreased vitality and longevity of the spermatozoa when immersed in these media. Although there is in practice no accurate method for determining the absolute vigor of movement of spermatozoa, an observer can readily ascertain when the movements of the spermatozoa cease. Cessation of movement makes the recognition of the death of

TABLE I
(A)

RABBITS INJECTED WITH SPERMATOZOA FROM RABBIT EPIDIDYMES

No. of Rabbit	(1) No. of Injections	(2) Time since Last Injection (Days)	(3) Experimental Results (Hours)		(4) Normal Control Results (Hours)	
			Vaginal	Serum	Vaginal	Serum
74A4	None		+6 −8	+ 7 −9		
	10	4	+2 −6	+ 4 −6	+9	+9
		11	+1 −3	+ 4 −5	+7	+6
B2A1	9	2	+2 −4	+ 3 −4	+8	+6
	3	7	+2 −4		+7	
D2	None		+7	+ 5		
	10	5	−4	−4	+8	+5
		12	−3		+4	
		20	−6	−5	+8	+7
53D2	None		+7	+ 6		
	10	3	−3	$-2\frac{1}{2}$	+6	+4
		23	−4	−3	+6	+7
		42	−4	−3	+5	+6
78X1	None		+9	+10		
	7	8	−1	−2	+4	+4
		18	−3	−2	$+5\frac{1}{2}$	+3
C19	None		+7	+11		
	3	8	−5	−3	+7	+6
	2	8	$-1\frac{1}{2}$	$-1\frac{1}{2}$	+4	+2
		40	−4	−4	+6	+6
		71	−4	−5	+6	+7
	3	72				
		10	$-\frac{1}{4}$	$-\frac{1}{2}$	+4	+3
		71	−4		+7	
U1	None		+5	+ 7		
	9	8	−3	−3	$+4\frac{1}{2}$	$+3\frac{1}{2}$
	3	8				
		8	$-\frac{1}{4}$	−1	+2	+4
		94	−4		+7	
D8	None		+8	$+9\frac{1}{2}$		
	7	10	−3	−2	$+5\frac{1}{2}$	+11
		67	−4	−4	$+6\frac{1}{4}$	$+5\frac{1}{2}$
D25	None		+8	+ 9		
	8	13	−3	$-\frac{3}{4}$	+7	+3
		61	−5	$-5\frac{1}{2}$	+7	$+7\frac{1}{4}$
		94	+4 $-4\frac{1}{2}$	−5		+8
	4	99				
		13	$-\frac{1}{3}$	$-\frac{3}{4}$	+3	+3

TABLE I —*Continued*
(A)

No. of Rabbit	(1) No. of Injections	(2) Time since Injection Last (Days)	(3) Experimental Results (Hours)		(4) Normal Control Results (Hours)	
			Vaginal	Serum	Vaginal	Serum
D24	None		+6	+ 7½		
	4	4	−4	−2½	+5½	+4
	7	16	− ¾	−1	+3¼	+4
	6	16				
		8	− ⅙	− ¼	+3	+3
D26	None		+7	+ 8		
	8		−4	−3	+6	+5
	3	16				
		4	−1	−1	+9	+9
	2	14				
		7		− ¾	+5¼	+3½

TABLE I—*Continued*
(B)

RABBITS INJECTED WITH EXTRACTS OF RABBITS' SALIVARY GLANDS

No. of Rabbit	(1) No. of Injections	(2) Time since Last Injection (Days)	(3) Experimental Results (Hours)		(4) Normal Control Results (Hours)	
			Vaginal	Serum	Vaginal	Serum
B6	None		+7	+8		
	14	4	− 7	−9	+7 − 8	+9 −11
		21	+6 − 6½		+6 − 6½	
B4	None		+7½ − 9¼	+9¼		
	11	10	+5 − 6		+5 − 6½	
		40	+7 − 7¾		+7 − 7¾	
C1	None		+9	+9		
	17	7	+7½ − 9½		+9½ −10	
C2	None		+5¾ − 6¾	+6¾ − 7¼		
	7	9	+6 − 6½		+6 − 6½	
		19	+7 − 8		+8 − 8¾	
	5	15	+9 −10		+9 −10	
C3	None		+8¼ − 9½	+9½ −11		
	11	13	+6 − 7¼		+6 − 8	
	9	18	+4¼ − 5¼		+4¼ − 5¼	
C4	None		+6¼ − 7¼	+7¼ − 9		
	8	7	+7 − 8		+8 − 8½	
	6	9	+9 − 9½		+8 − 9	
	10	36	+5¾ − 6¼		+5¾ − 6¼	
		52	+3 − 4		+3 − 4	
C6	None		+5¾ − 6		+5¾ − 6	
	7	5	+6½ − 7	+7 − 8¼	+6½ − 7	+7 −8½
	6	9	+5¾ − 6	+6 − 6¾	+5¾ − 6¼	+6 −6¾
	3	5	+8¼ − 9	+8 − 8⅚	+9 − 9¾	+8⅚ −9½
		34	+7⅓ − 8	+8 − 9	+7⅓ − 8	+8 −9

TABLE I—*Continued*

(C)

FEMALE RABBITS INJECTED WITH EJACULATE COMING FROM VASECTOMIZED MALES

No. of Rabbit	(1) No. of Injections	(2) Time since Last Injection (Days)	(3) Experimental Results (Hours)				(4) Normal Control Results (Hours)			
			Vaginal		Serum		Vaginal		Serum	
C5	None		+ 9	− 10	+ 10	− 12				
	7	5	+ 8	− $8\frac{5}{6}$	+ 7	− 8	+ 8	− $8\frac{5}{6}$	+ 8	− 9
	10	7	+ 6	− $7\frac{1}{4}$	+ 8	− 9	+ 6	− 7	+ $7\frac{1}{2}$	− $8\frac{1}{2}$
C7	None		+ $5\frac{3}{4}$	− $6\frac{1}{3}$	+ $8\frac{1}{3}$	− $9\frac{1}{2}$				
	7		+ $5\frac{3}{4}$	− $6\frac{1}{3}$	+ 6	− $6\frac{1}{3}$	+ $5\frac{3}{4}$	− $6\frac{1}{3}$	+ 6	− $6\frac{1}{2}$
	8	11	+ 7	− $7\frac{3}{4}$	+ 8	− 9	+ 6	− 7	+ 8	− 9
	4	12	+ 10	− $11\frac{1}{2}$	+ $11\frac{1}{2}$	− 13	+ 10	− $11\frac{1}{2}$	+ $12\frac{1}{2}$	− 13
C8	None		+ 4	− $4\frac{1}{3}$	+ $4\frac{1}{3}$	− 5				
	4	5	+ 5	− $5\frac{1}{3}$	+ $5\frac{1}{3}$	− $6\frac{1}{2}$	+ $5\frac{1}{3}$	− 6	+ $5\frac{1}{3}$	− $6\frac{1}{2}$
	5	6	+ $7\frac{1}{2}$	− 8	+ $8\frac{1}{2}$	− 10	+ 8	− $8\frac{1}{2}$	+ $8\frac{1}{2}$	− 10

EXPLANATION FOR TABLE I

(1) In this column are recorded: in Table A, the number of injections of spermatozoa coming from the epididymis; in Table B, the number of injections of salivary gland extract; and in Table C, the number of injections of sperm-free ejaculate.

(2) In this column, "Time since Last Injection," the time that elapsed between the last injection and when the "test" was made is indicated. As was previously stated, the injections were usually given at intervals of from 3 to 6 days. However, at other times a longer interval elapsed between series of injections. Whenever this was the case, the fact is indicated in this column.

(3) Spermatozoa were placed in solutions of vaginal secretions or serum of the treated rabbits. The plus sign (+) indicated that the sperm cells were still alive after the number of hours indicated. The minus sign (−) indicates that the sperm cells were found to be dead at the hours indicated.

(4) All, or nearly all, of the tests of (3) were checked with similar tests using serum and solutions of vaginal secretions of normal adult female rabbits. Spermatozoa coming from the same source were used in (3) and (4).

EXAMPLE

RABBIT C19.—A preliminary test, before any injections were given, showed that spermatozoa in a solution of her vaginal secretion were alive after 7 hours. Spermatozoa were still alive in the serum of the same rabbit after 11 hours, when observations were discontinued. The rabbit was then given three injections of spermatozoa. On the 8th day following the last injection tests were made on the effects of the vaginal secretions and serum upon spermatozoa with the results indicated. Two more injections were then given and on the 8th, 40th, and 71st days, respectively, similar tests were made. Then, beginning on the 72d day following the last previous injection, another series of three injections was started. Then, on the 10th and 71st days after the final injection, the usual tests with vaginal secretions and serum were again made.

the spermatozoa easy. There is a marked loss in vigor of the spermatozoa which are immersed in serum or a medium containing the vaginal secretion after a shorter or longer period of time. However, this condition is very much more pronounced in the case where the medium comes from rabbits which received injections of spermatozoa. Agglutination of the spermatozoa also comes on much more rapidly in such a medium.

The immunity to spermatozoa following a given number of injections of spermatozoa is apparently only temporary, for fertility returns again after a shorter or longer period of time after cessation of injections with spermatozoa. There is, however, evidence that if injections are continued at suitable intervals following the initial immunizing injections, the animal may remain in a refractory state to fertilization.

Aside from the fact that the rabbits in many cases, both control and experimental, did not eat for a few hours after the injections, their behavior and general well-being was not apparently affected by the injections. A record was kept of the weights of the animals during the different phases of the experiment. Only adult rabbits were used and there was no appreciable rise or fall in weight as a result of the treatment. There was no evident difference in sexual activity between the experimental, control, and normal animals. All the females were allowed to copulate with males whenever and as often as they would; they were given every opportunity to become pregnant. The experimental records show that the rabbits which received injections of spermatozoa mated practically as often as did the normal rabbits and those which received injections of salivary gland extracts or sperm-free ejaculated material from vasectomized males. However, the significant point is that those animals which were immunized to spermatozoa showed a prolonged period of infertility and a far greater percentage of fruitless copulations.

That the oestrous cycle in the female rabbit was not affected by the injections of spermatozoa was established by making periodic examinations of the ovaries and genital tracts of the treated animals. Laparotomies were performed on all of the treated animals. The presence of mature Graafian follicles, corpora hemorrhigaca, and corpora lutea gave evidence that ovulation was occurring in a normal

manner. Only in one case was an abnormality of the genital system found. This rabbit, B4, which had received control injections of salivary gland extract, had a cystic ovary on the right side about the size of a pigeon's egg. The ovary on the left side had a normal appearance.

Three rabbits, D25, D24, and D26, all of which had received immunizing injections of spermatozoa, had their sera and vaginal secretions tested for spermatotoxic properties and were then killed at different intervals following copulation with normal males and their genital tracts removed, opened, and examined for a study of the contained spermatozoa. Shortly (within 10 minutes) after copulation had occurred, samples of semen were removed from the vagina by the method already described to ascertain if normal quantities of active spermatozoa had been deposited there. In all three cases this had occurred. D24 was killed 16 hours after copulation. Although many dead spermatozoa could be recovered from her genital tract, not a single live spermatozoön was discovered. D25 was killed 12 hours after copulation, and an examination of the spermatozoa in the vagina and uterine tubes showed that all of them had lost their motility and were apparently dead. Large numbers of the spermatozoa were clumped together and around epithelial cells of the female tract. Only about 5 per cent of the spermatozoa were alive in samples taken from the genital tract of D26, which was killed $6\frac{1}{2}$ hours after copulation. These were found in the upper portion of the vagina below the cervix. All those spermatozoa which had found their way into the uterine tubes were immobilized. The genital tracts of two normal female rabbits which had copulated with the same male rabbits which mated with D24, D25, and D26 contained many active spermatozoa, 28 and 43 hours, respectively, after copulation.

EXPERIMENT 2

SPERMATOTOXINS FROM INJECTIONS OF RABBIT TESTES EXTRACTS

Twelve female rabbits were used in this study to determine the antigenic effects of the repeated injections of Ringer's solution extracts of rabbit testes. Since male rabbits were castrated to furnish the spermatozoa used in the immunizing injections in Experiment 1,

the testes of these same rabbits were available for injection into the animals used in Experiment 2. The rabbits were given injections in all respects similar to those given in Experiment 1, with the exception that testicular material rather than material from the epididymis served as the antigen. Prior to the injections, all the rabbits in this series had given birth to young. The use of originally fertile stock was thus assured. Since the injections were given on corresponding days and at corresponding intervals, the same animals that were used for controls in Experiment 1 were used as controls in Experiment 2. The results of this latter study are summarized in Table II.

The results of this experiment are essentially like those of Experiment 1. Consequently, any general remarks that were made for that experiment are applicable to this experiment. The repeated injection of fresh testicular extracts of rabbit testes produces antigenic effects in the injected rabbits, evidenced by the appearance in the blood and vaginal secretions of a substance or substances which will rapidly bring about the destruction of rabbit spermatozoa both *in vivo* and *in vitro*. That the temporary sterility induced by the immunizing injections of the testicular extracts occurs without any apparent effect on the oestrous cycle is borne out by the fact that the rabbits so treated do not react differently sexually from normal or the control animals. The cyclic changes in the ovaries of the rabbits used in this experiment indicated that ovulation was occurring in a normal manner.

Three female rabbits, still actively immunized to rabbit spermatozoa, were killed at various intervals after copulation to determine the condition of the spermatozoa in their genital tracts. Before the rabbits were killed, however, preliminary tests were made to ascertain whether the deposited spermatozoa were normal as regards vigor of movement. These tests in all three cases were positive. The results were as follows: Rabbit C_9, killed $4\frac{1}{4}$ hours after copulation: with the exception of about 2 per cent, all the spermatozoa in the genital tract were immotile; those that were alive were found in the upper third of the vagina. Rabbit C_{27}, killed 7 hours after copulation: only dead spermatozoa could be found.

The remaining six rabbits were kept on hand in the laboratory to determine the duration of sterility (Table V).

TABLE II

Female Rabbits Injected with Testicular Extract from Rabbits[1]

No. of Rabbit	(1) No. of Injections	(2) Time since Last Injection (Days)	(3) Experimental Results (Hours)				(4) Normal Control Results (Hours)			
			Vaginal		Serum		Vaginal		Serum	
D1	None		+ 9	− 10	+ 10	− 13				
	8	8		− 6		− 6	+ 12		+ 12	
	3	4		− 1		− ¾	+ 4		+ 4	
		13	+ 1	− 1⅓		− ¾	+ 6		+ 3	
		34	+ 5	− 8		− 6	+ 8		+ 8	
78X3	None		+ 9	− 11	+ 10	− 11				
	8	9		− ⅓		− ⅔	+ 5		+ 4	
D9	None		+ 8		+ 8					
	9	8	+ 2	− 2⅔		− 2	+ 5		+ 6	
		56	+ 5	− 7	+ 6⅓	− 8	+ 9		+ 9	
C26	None		+ 8⅓		+ 8⅓					
	6	26		− 4		− 2½	+ 9		+ 4	
		46	+ 6	− 7			+ 9			
		68	+ 5½	− 6		− 6	+ 5½	− 6	+ 6	− 6⅔
B27	None		+ 11		+ 11					
	6	8				− ⅓			+ 4⅓	
	5	6		− ⅓		− ⅔	+ 5½		+ 5½	
		23		− 7		− 4¾	+ 9		+ 7	
		66	+ 8	− 8½	+ 6	− 7	+ 10		+ 10	
C15	None		+ 7¾		+ 7¾					
	6	13		− 2		− 1⅓	+ 8		+ 4½	
	5	9		− ⅙		− ⅙	+ 3		+ 3	
		73	+ 6	− 7⅓	+ 5	− 5⅚	+ 10		+ 7⅓	
C9	None		+ 7⅓		+ 5⅚					
	6	9		− ½		− ½	+ 5		+ 5	
		14	+ 1	− 1⅕		− 1	+ 4		+ 4	
C27	None		+ 5⅚		+ 5⅚					
	4	5		− 2		− 1⅙	+ 4		+ 3¾	
	5	6		− ½		− ½	+ 3½		+ 3½	
		22	+ 4⅓	− 4¾		− 4	+ 5⅚		+ 8	
C16	None		+ 9		+ 9					
	6	4		− 1⅙		− 1¾	+ 4		+ 4	
		19		− 2		− 1½	+ 5⅓		+ 4⅙	
		31	+ 5½	− 6	+ 4	− 4⅓	+ 7½		+ 5½	
C21	None		+ 6		+ 6¾					
	6	5		− ½		− ⅓	+ 5		+ 4	
		13	+ ¾	− 1	+ ¾	− 1	+ 4		+ 4	
C14	None		+ 6	− 9⅓	+ 9⅓					
	8	6		− ⅙		− ⅙	+ 3		+ 3	
C29	None		+ 6¾		+ 6¾					
	3	8		− ¼		− ⅙	+ 4⅓		+ 2¾	
	5	3		− ½		− ¼	+ 7		+ 5	
		7	+ 1	− 1¾		− ¼	+ 5⅔		+ 5⅔	

[1] Experimental controls for this series are the same as those of Experiment 1 (B and C).

EXPERIMENT 3

SPECIFICITY OF SPERMATOTOXINS

This experiment was intended to show whether the spermatotoxins for rabbit spermatozoa, induced by the injections of rabbit spermatozoa into female rabbits, are specific for rabbit spermatozoa or whether they would also exert a toxic action upon the spermatozoa from the albino rat.

Preliminary tests showed that normal rabbit serum is a less favorable medium for the life of rat spermatozoa than is the serum coming from rats. To show whether this innate characteristic for rabbit serum to hasten the death of rat spermatozoa is enhanced by immunizing the rabbit with rabbit spermatozoa, the following procedure was carried out: The epididymis of an adult albino rat was aseptically removed from the testis and finely sliced, then shaken by hand in 10 cc. of Ringer's solution, and then filtered through four thicknesses of cheesecloth. A loopful of such a suspension of spermatozoa was then added to the rabbit sera contained in depression slides. The sera used were those coming from rabbits which had received injections of rabbit spermatozoa (Experiment 1) and those coming from normal rabbits which were used as controls. Reciprocal tests were also made in which rabbits were given injections of rat spermatozoa to determine if a serum for rat spermatozoa would likewise be toxic for rabbit spermatozoa.

The results of this study are summarized in Table III, and show that spermatotoxins induced in the female rabbit by the injection of rabbit and rat spermatozoa are not species specific, since they react not only to spermatozoa of the kind injected but also to the spermatozoa of the rat and rabbit, respectively. The rabbits which had been given injections of rat spermatozoa suffered a noticeable fall in weight. One of these rabbits died of anaphylaxis within 4 minutes after an injection had been given. Injections had to be discontinued in two other rabbits because of their extremely emaciated condition. These latter rabbits are not listed in Table III. When the injections of rat spermatozoa were discontinued, the rabbits soon regained their weight, and in the course of time (Table V) gave birth to young. It is of interest to note that the injection of rat spermatozoa into female rabbits was not followed by the prolonged period of infertility

which characterized the group of rabbits which received injections of rabbit spermatozoa and extracts of rabbit testes.

TABLE III

SPECIFICITY OF SPERMATOTOXINS

No. of Rabbit	Antigen Used	No. of Injections	Time Required for Serum to Immobilize Spermatozoa (Hours)	
			Rabbit	Rat
B2A1	rabbit sperm	12	+ 2 − 4 Cont. +10	+2 −2½ Cont. +4½
74A4	rabbit sperm	10	+ 2 − 3 Cont. +15	+1¼ −1½ Cont. +2 −3
53D2	rabbit sperm	10	− 1 Cont. +12	+3½ −4¼ Cont. + 6
D2	rabbit sperm	10	+ 2 −4 Cont. +12	+2 −3 Cont. +3 −4¾
C19	rabbit sperm	8	+ ½ −1 Cont. +15	+4 −5 Cont. +6 −7
C10	rat sperm	11	+ 7 −9 Cont. +12	+1 −1¼ Cont. +5 −5½
P54A5	rat sperm	7	+ 7½ −8½ Cont. +13	+ ¼ − ½ Cont. +2¼ −3¼
C11	rat sperm	13	+ 4 −7 Cont. +11	+ ¼ − ¾ Cont. +2 −2½
C43	rat sperm	10	+ 7 −9 Cont. +13	+ ¼ − ½ +4 −4½

EXPLANATORY EXAMPLE

Rabbit B2A1 had her serum tested for toxic activity for rabbit and rat spermatozoa, respectively, after she had received twelve injections of rabbit spermatozoa. Rabbit spermatozoa immersed in this serum were yet alive after 2 hours but were dead after 4 hours. Rat spermatozoa were alive after 2 hours but dead after 2½ hours. Serum from a normal adult female rabbit was used as a control. In this control, rabbit sperm were yet alive after 10 hours, and rat spermatozoa were likewise alive after 4½ hours. The spermatozoa used in the control tests were of the same source as those in the experimental tests.

EXPERIMENT 4

DEVELOPMENT OF SPERMATOTOXIC SUBSTANCES AFTER INTRAVAGINAL INJECTIONS OF SPERMATOZOA

Five female rabbits were given a large number of intravaginal injections of rabbit spermatozoa. Whenever these rabbits came in oestrum, they were allowed to mate with males as often as they would. Naturally, while the animals were out of oestrum it rarely happened that spermatozoa could be deposited into the vagina by natural means. A group of from eight to twelve normal adult female rabbits was kept on hand in the laboratory throughout the experiment for breeding purposes. Whenever any of these normal animals came in heat, they were allowed to copulate with normal males. The semen was then collected by means of a pipette from the vaginas of these rabbits and immediately distributed to the vaginas of the experimental animals. This process was repeated as often as possible. In this manner it was an easy matter to collect comparatively large quantities of semen. However, there were times when no matings would occur for periods of 10 days or longer. At such times when it was desired to continue the intravaginal injections, normal male rabbits were castrated, and their spermatozoa, coming from the macerated epididymis, suspended in Ringer's solution, injected directly into the vagina. Table IV gives evidence that such repeated administrations of spermatozoa produce antigenic effects. Table V shows that in a number of cases the immunity induced by the intravaginal injection of spermatozoa manifests itself by increased number of sterile copulations. However, no prolonged period of infertility followed the intravaginal injections of spermatozoa. The results of this experiment substantiate the contentions of Waldstein, Ekler, and McCartney that the presence of spermatozoa in the genital tract of the female may exercise antigenic effects. But there is hardly enough evidence that such repeated intravaginal injections of spermatozoa will lead to a sufficiently high antibody production to cause sterility.

It seems improbable that the same process—deposition of spermatozoa into the female genital tract—which is one factor for the perpetuation of the species, should also tend to produce the opposite effect, namely sterility, as a result of antibodies produced against spermatozoa.

It may be that the few cases of delayed fertility, evidenced by an increased number of sterile copulations, following the repeated injections of spermatozoa into the vagina may be accounted for by the entrance of spermatozoa into the circulation through abrasions in

TABLE IV

EFFECTS OF INTRAVAGINAL INJECTIONS OF RABBIT SPERMATOZOA

No. of Rabbit	(1) No. of Injections	(2) Time since Last Injection (Days)	(3) Experimental Results		(4) Normal Control Results	
			Vaginal	Serum	Vaginal	Serum
U2	None		+ 7¼	+7¼		
	24	3	+ 6 − 6⅓	+7 − 7½	+ 6⅓ − 7	+ 7½ − 8½
	11	7	+ 5 − 6¼		+ 6⅔ − 7	
C17	None		+8⅓			
	4	1	+5⅔ − 6		+ 6 − 6½	
	24	6	+6¾ − 7⅔		+ 7½ − 8	
		8	− 8	+ 8 − 9	+ 8 − 8½	+ 8 − 9
	4	7	+4½ − 5	+ 3¾ − 4¼	+ 5 − 5¾	+ 6 − 6¼
		16	+6 − 6⅙	+ 5½ − 6½	+ 6⅙ − 6½	+ 8 − 8¾
B20	None		+7½	+ 7½		
	31	1	+5¾ − 6⅙	+ 5 − 5¾	− 6⅙	+ 5¾ − 6⅙
	8		+7⅙ − 8		+ 8½ − 8⅚	
	49	2	+5 − 5¾	+ 7¾ − 8¾	+ 6½ − 7¾	+ 9¼
		9	+3 − 4⅙	+ 4½ − 5	+ 5 − 6¼	+ 5 − 5¼
D21	None		+5¾	+ 6½		
	19	2	+6 − 7	+ 7½ − 8	+ 7½ − 8	+ 8½ − 9⅓
		7	+4¾ − 5¼	+ 3 − 3⅓	+ 5½ − 6	+ 3⅓ − 4
	11	5	+4⅔ − 5	+ 3 − 4	+ 6 − 7	+ 4½ − 4⅔
		11	+5⅓ − 6¼	+ 2 − 3⅙	+ 6½ − 7¼	+ 4 − 5¼
D27	None		+6	+ 6		
	8	½	+4 − 5	+ 5 − 6	+ 4 − 5	+ 5 − 5¾
	3	4	+2⅚ − 3	+ 3 − 4¼	+ 3½ − 4¼	+ 4¼ − 4¾
	27	1	+4 − 4¾	− 4	+ 5 − 5¼	+ 4¾ − 5½
		6	+6 − 6½		+ 7 − 7½	
	26	½	+7 − 8	+ 6 − 7	+ 9	+ 8 − 9
		14	+4¾ − 5½	+ 7⅓ − 8	+ 4¾ − 5¼	+ 8½ − 9
D28 (prostate-gland control)	None		+8	+ 8		
	34	½	+6 − 6¾	+ 6¼ − 6¾	+ 6 − 6¼	+ 6¾ − 7¼
	11	1	+5⅙ − 6	+ 5⅙ − 6	+ 6½ − 7	+ 7 − 7½
		7	− 4	− 4	+ 4 − 4¾	+ 5 − 6
		22	+6⅓ − 6¾	+ 7¼ − 7⅔	+ 6¾ − 7¼	+ 7⅔ − 8¼
D34 (prostate-gland control)	None		+6 − 6¾	+ 8 − 9		
	12	1	+4½ − 5		+ 5¼ − 5¾	
	27	3	+3⅚ − 4	+ 4 − 5	+ 5 − 5½	+ 5½ − 6
	28	3	+6 − 6¾	+ 5 − 6	+ 7 − 7¼	+ 6¾ − 8⅓
	7	1	+6⅔ − 7¼		+ 8¾	
		15	+11 −13	+ 9½ −11	+11½ −13	+12 −13

the genital mucosa as a result of the artificial means used at times to introduce spermatozoa into the vagina.

TABLE V

EFFECT OF SPERMATOTOXINS ON FERTILITY OF FEMALE RABBITS

No. of Rabbit	Before Injections			Injections		After Injections			
	No. of Sterile Matings	No. of Litters Born	Average No. of Young per Litter	Material Injected	No. of Injections	No. of Sterile Matings	Time until Return of Fertility (Weeks)	No. of Litters Born	Average No. of Young per Litter
74A4	0	3	7.7	Spermatozoa of rabbits	10	4	17	1	5
B2A1	1	3	7.3		12	5	25	.....	.....
D2	0	4	7.0		10	3	22	2	4.5
53D2	0	2	6		10	6	25	.....	.....
78X1	0	1	8		7	3	12	1	7
C19	1	3	7		13	4	13	2	3.0
U1	0	2	6.5		12	2	9	1	6.0
D8	0	1	4		7	2	11	2	4
D1	0	2	5.0	Extract of rabbit testis	11	3	7	1	1
78X3	0	1	7		14	4	9	1	6
D9	1	4	7.8		9	3	6	2	4
C26	0	2	6		6	4	14	1	6
C15	2	3	8		16	3	19	.....	.....
B27	0	4	6		10	2	11	1	5
B6	0	4	6.5	Rabbit salivary-gland extract	14	1	3	3	6
B4	1	5	7.6		11	0	1	4	8
C1	0	1	4		17	0	4	1	4
C2	0	2	6.0		12	1	2	2	7
C3	0	1	5		20	0	3	1	5
C4	1	2	6.5		14	1	1	3	5.7
C6	0	3	8.3		16	0	3	1	8
C5	2	4	5	Sperm-free ejaculate	17	0	1	2	6.5
C7	0	2	7.5		19	0	3	3	6.3
C8	0	1	4		15	0	3	1	5
C10	0	3	6.6	Spermatozoa of rats	11	0	2	3	5
54A5	0	1	4		7	2	4	1	3
C11	0	2	6.5		15	3	5	1	6
U2	0	3	5.7	Rabbit spermatozoa by intravaginal route	68	2	3	4	6.2
C17	1	4	4		40	0	1	1	5
B20	2	5	5.8		80	1	2	1	4
B21	0	2	6.5		26	2	3	2	7
D27	0	3	9		64	3	3	1	6

The experimental controls for this series were two adult female rabbits which were allowed to copulate with two vasectomized male rabbits during their periods of oestrum, and which at other times

were given intravaginal injections of the sperm-free ejaculate as it was normally deposited by the vasectomized males into other females.

SUMMARY OF BREEDING EXPERIMENTS

In no case was it found that the injection of rabbit spermatozoa or fresh testicular extract into female rabbits had an effect on the the oestrous cycle. This was true also after injections of rabbit salivary gland, the ejaculated material coming from vasectomized rabbits, and rat spermatozoa. Table V shows that there may be a very noticeable delay in return of fertility in female rabbits following the intravenous or intraperitoneal injection of rabbit spermatozoa or fresh extracts of the rabbit testis. In the column headed "Time until Return of Fertility" the time, in weeks, that elapsed between the beginnings of injections and the first successful or fertile mating is recorded. The period of sterility following the injection of rabbit spermatozoa or testicular extract may have a duration of 6–25 weeks. Three rabbits have not become pregnant up to the time of writing (26 weeks after the beginning of injections), even though they have repeatedly copulated and have shown the familiar signs of oestrum. Laparotomies have been performed on these three rabbits at various intervals during their sexual cycles, and the presence of mature follicles and corporea lutea showed that ovulation had occurred in a normal manner. When one considers that even in a group of normal female rabbits there may be considerable variation in the time when certain individuals come into oestrum, copulate, and afterward deliver young, it is not surprising that even among the controls not all rabbits became pregnant immediately following the injections of salivary-gland extract. Table V does show, however, that the injections of rabbit spermatozoa or testicular extracts into female rabbits may delay the return of fertility far beyond the time that could be accounted for by normal variation and the mere injection of substances foreign to the blood stream.

SUMMARY

1. The serum and vaginal secretion of a female rabbit injected with rabbit spermatozoa or fresh testicular extract become toxic for rabbit spermatozoa.

2. The longevity of spermatozoa normally deposited in the genital tract of the female rabbit is greatly decreased by the previous intravenous injection of rabbit spermatozoa or testicular extract.

3. Infertility for a period of 6–25 weeks may follow the repeated injection of spermatozoa or testicular extract into female rabbits. The injection of rabbit salivary-gland extract, or the sperm-free ejaculate coming from vasectomized males, into female rabbits does not cause sterility.

4. The injection of rabbit spermatozoa or fresh testicular extracts into female rabbits does not affect the oestrous cycle. However, if the injections are given during pregnancy, abortion or resorption of the fetuses may sometimes occur.

5. The serum of a rabbit given intravenous injections of rabbit spermatozoa also becomes toxic for rat spermatozoa. Similarly, the serum of a rabbit immunized to rat spermatozoa becomes toxic for rabbit spermatozoa.

6. The repeated intravaginal injection of rabbit spermatozoa, by natural and artificial means, in the female rabbit may produce antigenic effects in the blood and vaginal secretions of the rabbit. However, evidence that sterility may ensue as a result of these intravaginal injections of spermatozoa is inconclusive.

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HYDRATION AND DEHYDRATION IN THE LIVING CELL. I. THE EFFECT OF EXTREME HYPOTONY ON THE EGG OF NEREIS

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FOR several years the writer has been engaged in the study of the effects of hypotonic sea-water on the uninseminated and the inseminated ova of marine animals (Just, 1920, 1922*a*, 1922*b*, 1923*c*, 1926). This study has led to conclusions of some importance for the analysis of the fertilization-reaction as well as of the division of the cell body in mitotic cell division. Much of this work has been done on the ova of *Echinarachnius* and of *Arbacia*. Various other ova, however, have been used—among others, those of *Nereis* and of *Platynereis*.

The ova of these annelids differ markedly from those of the two echinids mentioned above: first, they are "ripe" for insemination while in the germinal vesicle stage, in which stage they remain intact after coming into sea-water until death unless inseminated; secondly, they respond to insemination not merely by separating their vitelline membranes but by extrusion of a cortically located jelly (Lillie, 1911, 1912; Just, 1912, 1915*a*, 1915*c*); and thirdly, during part of the long period—45 minutes in the case of the egg of *Nereis* (Lillie, *loc. cit.*) and 25 minutes in the case of the egg of *Platynereis* (Just, 1915*c*)—in which the spermatozoön remains external to the ovum, they undergo a series of cortical changes. Because of these differences, the writer undertook experiments on the effects of hypotony on these ova with the purpose of analyzing the nature of their cortical response to insemination. The account that follows, though referring almost exclusively to ova of *Nereis*, is equally true of those of *Platynereis*. The observations and experiments were

made during several summers at the Marine Biological Laboratory, Woods Hole, Massachusetts, where an abundance of worms may be had during each month from full to new moon (Lillie and Just, 1913; Just, 1914).

But the present report, the first of a series dealing with the effects of hypo- and hypertony on animal cells—including marine ova, protozoan cells, and cells from the adult tissues of both invertebrates and vertebrates—has a deeper significance for a fundamental problem in the biology of the animal cell. I refer to the question concerning the mechanism of water-holding and water-output by viable cells. Despite the vast amount of literature on the permeability of cells there is no adequate description of the movement of water *within* the cell. The present series of communications aims to present such descriptions. If these observations of mine be confirmed and extended, they may be found to be of great importance for the analysis of the rôle of water in cell physiology, and perhaps of the physical structure of protoplasm. In a paper read before the American Society of Zoölogists, December, 1926, I referred to one phase of this problem. In the present paper, experiments and observations dealing largely with an extreme degree of hypotony are set forth; this is done to establish criteria of death-changes in such hypotony *not to be confused with effects of less degrees of hypotony in which the cells remain viable.* Death-changes manifested by cells in distilled water or other brutal and lethal solutions are interesting, but the already sufficient notices of such changes found in the literature would make this paper superfluous if published for this point alone.

EXPERIMENTAL

We may begin with the consideration of the effects of hypotony on the uninseminated eggs of *Nereis limbata.*

A. THE WATER-HOLDING CAPACITY OF THE UNINSEMINATED EGG OF NEREIS

Uninseminated eggs of *Nereis* exposed to dilute sea-water take up water and increase in volume until they come into osmotic equilibrium with the dilution; depending upon the degree of dilution, they remain intact for a time and then cytolyze. In the case of extreme dilutions the eggs break down before they reach equilibrium. Thus, the history and fate of these eggs are similar to those de-

scribed by R. S. Lillie for the sea-urchin egg. There is a notable difference, however: the egg of *Nereis* can withstand longer exposure to more extreme dilutions. This is shown in what follows. I present first a description of the effects of the most extreme dilutions on the uninseminated egg of *Nereis*.

The uninseminated eggs of *Nereis*, when exposed to the action of distilled water (or tap water, as they were in the first experiments), break down before they reach osmotic equilibrium. The time to complete disintegration runs from 180 to 240 seconds in different lots of eggs. A protocol of a single experiment made on eggs from 6 females gave the figures shown in Table I.

This experiment is typical of results on eggs from over 100 females used during several seasons at Woods Hole. Determination

TABLE I

RATE OF DISINTEGRATION OF UNINSEMINATED *Nereis* EGGS IN DISTILLED WATER

No. of Female	1	2	3	4	5	6
Time in seconds to complete disintegration in distilled water.....	180	210	180	240	180	240

of the time to cytolysis of uninseminated eggs became part of the routine work on the effects of hypotony; hence the data on this point are most complete. Deviations from the figures given in Table I could always be accounted for. Thus, eggs from females kept in the laboratory for more than 24 hours are extremely variable and never so resistant as those from animals used the day following the night of capture. Eggs used from animals immediately after capture are no more resistant, however, than those taken from the animals the morning after capture. Therefore, most of the experiments were on such eggs. I found it best to use the same method throughout—namely, to mount a drop of eggs under the microscope in 10 cc. of dilute sea-water. The whole process could then be easily followed, the time of cytolysis being recorded by means of a stop watch. Approximately the same number of eggs in the same volume of dilute sea-water was used in every experiment.

The figures given reveal a high resistance of the egg of *Nereis* to

sea-water of extreme dilution. This resistance is the highest among eggs studied and is of significance, as later studies on the effect of hypotony on this egg will show. Observation also showed that the egg responds to extremely dilute sea-water in a way somewhat different from that described for eggs of other forms studied.

The first well-defined effect of exposing the egg of *Nereis* to distilled water is the separation of the vitelline membrane. This takes place about 1 minute after exposure. Usually a dark-brown spot appears in the cortex, and from this the membrane begins to separate from the egg. A wave now passes inward from the membrane, leaving the widened cortex more sharply defined. The cortex is clearly revealed as made up of fine vesicles. The egg now sends out minute drops in linear arrangement so that it bristles with what appear as fine threads, resembling the condition found in the normal uninseminated egg of *Phascolosoma*, as described by Gerould, whose description I have verified in my work on this egg. In suspensions of Chinese ink in sea-water one can determine that exposure to distilled water induces jelly extrusion. Thus, each egg is inclosed in a jelly hull. Between 2 to 3 minutes after exposure the endoplasm swells and obliterates the remnant of cortex, thus coming to lie against the vitelline membrane. The whole egg, now brown, rapidly turns black. Then the endoplasm fades leaving a transparent mass within the membrane.

This observation is suggestive. In the first place, this effect of distilled water in setting up cytolysis which spreads as a wave is by no means novel. In addition to my own observations on eggs of other forms, there are some old ones of Sollmann which show the same effect. Speaking of the effect of distilled water on the freshly exercised ova of *Asterias*, Sollmann says: "Suddenly a wave passes over the cell from one side to the other; everything dissolves except a portion of the spongioplasm, which flows together in a stringy, contorted, waxy mass, or into larger globules." These death changes recall the granular degeneration or *tropfige Entmischung* of Albrecht.[1]

[1] Albrecht (*Verhandlungen der deutschen pathologischen Gesellschaft* [1900]) regards protoplasm as an emulsion which under the influence of various agents—water, for example—changes to a mechanical mixture which contains drops of one substance suspended in another. Other lethal influences bring about the same appearance. Thus, Verworn described droplet formation particularly in the completely hyaline marine

Similar granular degeneration has been described by Verworn in protozoan cells due to injury by cutting.

The wave of cytolysis in the egg of *Nereis* would seem to indicate a susceptibility gradient, which, however, is certainly not related to polarity, because the uninseminated egg—in the germinal vesicle stage—exhibits the initial sign of cytolysis at any point, with reference to the polar axis, indicated by the elongation of the germinal vesicle at right angles to the long axis of the cell. In uninseminated mature echinid ova the nucleus lies in any position with reference to the jelly funnel; and in the egg of *Asterias*, after maturation, according to Yatsu, the nucleus does not lie in any fixed position. The last point may be of no great significance, since after maturation the egg of *Asterias* rapidly loses fertilization power, hence is not wholly normal. But in the egg of *Nereis*, at least, this wave of susceptibility is evidence of a cortical not a polar gradient.

In the second place, this observation is of interest because it reveals that droplets come out of the cortex which form strands on the outside. This may offer a hint concerning the normal mechanism of jelly extrusion. As is well known from the original observations made by Lillie, the cortex of the egg of *Nereis* is made up of alveoli which contain this jelly. This striated cortex is not a zona radiata, as Wilson erroneously described it. On insemination, by treatment with hypertonic sea-water (Lillie, 1911), or during exposure to increased temperature (Just, 1915*b*), the egg extrudes this jelly. No attempt, however, has ever been made to explain how this jelly, if it exist as such, is able to get through the vitelline membrane. It may be that normally the jelly pours out through perforations in the vitelline membrane. This is not so far-fetched as might at first

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form, *Hyalopus Dujardinii*. If a clear pseudopodium which is perfectly free of granules be cut off with a knife, it undergoes granular disintegration. See too Verworn's description of death-changes following cutting in *Spirostomum* (cf., also, overstimulation of *Pelomyxa* on one side). Concerning the former, Verworn says: "Death can be followed by the eye, and its course resembles that of a spark that passes over a fuse and leaves behind it merely a loose mass of ashes. It creeps over the whole body, seizing upon particle after particle, surprising cilium after cilium in normal activity, and forcing them directly from active life to a standstill, until that which a moment before was in active motion is changed into a dead mass of granules" (Verworn, *General Physiology*, translated by F. S. Lee [The Macmillan Co., 1899]).

seem, in view of the fact pointed out above that the egg of *Phascolosoma* normally bristles with cortical projections that pierce the membrane. However, so far I have been unable to determine, under either dark or bright field, that the membrane around the egg of Nereis is fenestrated. But this may mean that normally the perforations are very minute. Certainly, as near as I can determine, normally the jelly does not come out in fine drops as if through holes. It would be interesting if this membrane should prove to be fenestrated and, indeed, if this be true for vitelline membranes generally.

Experiments were also made with sea-water of lesser dilutions than that given above. Of these, the dilutions made up of 95 and 90 parts distilled water plus 5 and 10 parts sea-water, respectively, gave

TABLE II

RATE OF DISINTEGRATION OF INSEMINATED *Nereis* EGGS IN DILUTE SEA-WATER

No.	Parts Distilled Water	Parts Sea-Water	Time in Seconds to Complete Cytolysis			
			1	2	3	4
1.....	100	0	200	185	180	200
2.....	95	5	240	300	185	215
3.....	90	10	210	220	200	200
4.....	85	15	780	720	980	1,020

about the same results as distilled water alone. The dilution 85 parts distilled water plus 15 parts sea-water is less harmful. This is shown by the following:

Eggs from each of four females are out to clean sea-water. Samples of each lot of eggs were exposed to dilute sea-water, and the time to complete cytolysis was noted. Tabulated results appear in Table II.

The time recorded is for cytolysis of the last egg; the figures are, therefore, the time for 100 percent cytolysis, as in the case of the figures previously given for the effect of distilled water alone. If, instead, we take the time of cytolysis for the first egg or for the majority of the eggs, the differences are of the same order. There is thus a decided resistance to the action of 15 percent sea-water.

In the dilution 80 parts distilled water plus 20 parts sea-water, at least 50 per cent of the eggs remain intact for 24 hours, even in

cases where the control eggs in sea-water have all cytolyzed. Thus, during July 3 and 4, 1926, I found that eggs placed in this dilution were mostly intact after 24 hours. Eggs from 4 females showed the following percentage intact: 63, 54, 41, and 57. This experiment repeated later during the season gave the same results. My experience during 1925 was similar.

In the dilutions 75 and 70 parts distilled water plus 25 and 30 parts sea-water, respectively, no eggs cytolyze in 24 hours. No citations from my notes are necessary on this point. These dilutions and that of 65 parts distilled water plus 45 parts sea-water were used throughout the seasons of 1925 and 1926, especially in connection with experiments to be reported in a later communication.

Eggs of *Platynereis*, cut out of the female, react in much the same way as those of *Nereis*.

These data reveal that the water-holding capacity of the uninseminated egg of *Nereis* is very great. Comparison with the egg of *Arbacia*, for example, is instructive. R. S. Lillie found that the degree of dilution required to cytolyze the majority of unfertilized eggs of *Arbacia* in $\frac{1}{2}$ hour is about 60 volumes per cent (60 volumes of tap water plus 40 of sea-water). In such a dilution the eggs of *Nereis* remain intact as long as, or longer than, eggs in sea-water. To bring about cytolysis in the majority of *Nereis* eggs in $\frac{1}{2}$ hour would require a dilution of between 80 and 85 parts distilled water plus sea-water to make the volume 100.

In the dilutions 80, 75, and 70 parts distilled water plus 20, 25, and 30 parts sea-water, respectively, a certain small percentage of the eggs on return to sea-water instead of *returning to the normal condition of the uninseminated egg* differentiate without cleavage, a still smaller percentage, reaching an abnormal swimming stage. These developing eggs are those which in the dilute sea-water separate membranes and extrude jelly. The dilutions 80 and 75 parts distilled water plus 20 and 25 parts sea-water, respectively, are best for this purpose. Fewer eggs show any development beyond membrane separation in the dilution 70 parts distilled water plus 30 parts sea-water. The length of exposure is a factor in the production of this differentiation without cleavage and of abnormal swimming forms.

The effect of dilute sea-water was also studied on the inseminated egg of *Nereis*. In the next section data are presented on the capacity of the egg to hold water during the period just after insemination.

B. WATER-HOLDING CAPACITY OF THE EGG OF NEREIS IMMEDIATELY FOLLOWING INSEMINATION (PERIOD OF MAXIMUM SUSCEPTIBILITY TO HYPOTONY)

For about 25 minutes after insemination the egg of *Nereis* is highly susceptible to the action of dilute sea-water. The data are remarkably uniform on this point, as Table III made from one of a large number of experiments during three seasons at Woods Hole shows.

Table III scarcely needs comment. It is at once obvious that after 24 minutes following the mixing of eggs and sperm, the eggs

TABLE III

RATE OF DISINTEGRATION OF INSEMINATED EGGS OF *Nereis* IN DISTILLED WATER (EGGS FROM 5 FEMALES)

Minutes after Insemination When Placed in Distilled Water	2	4	6	8	10	12	14	16	18	20	22	24	26	28
Time (in seconds) to disintegration in the distilled water—														
No. 1	30	15	20	30	30	40	40	30	60	60	60	60	100	180
No. 2	10	15	20	20	60	30	40	30	40	60	60	180	300	300
No. 3	15	10	20	30	30	20	40	40	45	40	60	180	300	300
No. 4	30	30	15	20	30	30	35	40	40	30	60	90	240	...
No. 5	30	20	15	20	20	60	30	...	40	30	40	90	180	...

previously susceptible to the action of the distilled water enter a period of resistance. The eggs used in all experiments were in the best possible condition, since in the inseminated controls 100 percent cleaved at practically the same time and since vigorous larvae were produced. Many experiments were made at night immediately following capture of the worms when the eggs should be at their best. It was found to be an easy matter to get fairly constant results provided the same method was employed throughout. Thus, in all experiments inseminations were made in 250 cc. of sea-water, using eggs from a single female. A drop of these eggs was carried over to 10 cc. of distilled water mounted under the low power of the microscope. The sperm suspension always freshly made up was the mini-

mum concentration that would insure insemination in all eggs. Polyspermy was carefully avoided. Every possible source of error was eliminated. The conclusion is thus warranted that following insemination the eggs of *Nereis* are highly susceptible to the action of dilute sea-water, as shown by their more rapid rate of disintegration when compared with uninseminated eggs. This period of extreme susceptibility lasts for about 25 minutes. Then the eggs return to a state of increased resistance which is even greater than that of uninseminated eggs.

Experiments were also made in this wise: Eggs were inseminated in normal sea-water and at intervals of 5 seconds up to 25 minutes after insemination were placed in distilled water for 10, 20, and 30 seconds and returned to normal sea-water. Such experiments consistently showed that for about 25 minutes after insemination the eggs are extremely susceptible, as revealed by their failure to cleave. Even a 10 second exposure is sufficient to cut down the cleavage percentage to zero. And this too where there are no visible signs of injury at the time of transfer. An objection to this type of experiment might be raised: exposed to distilled water for a short time, the eggs, though they show no evidence of injury, nevertheless fail to cleave on return to normal sea-water because of the rapid loss of water by the water-logged cells on return to sea-water.

Inseminated eggs of *Platynereis* behave similarly. The animals were allowed to copulate (Just, 1914); and at intervals after egg-laying, eggs were exposed to distilled water. In this way a series of exposures was established. The period of susceptibility lasts for 16 minutes after egg-laying.

Comparison of the period of susceptibility in the inseminated egg of *Nereis* with that of *Echinarachnius* is instructive. In the egg of *Echinarachnius* this period is of *seconds'* duration, whereas in the egg of *Nereis* it is of *minutes'* duration. I should say that the period is about 100 times longer in the egg of *Nereis* than in that of *Echinarachnius*. In the egg of *Echinarachnius* this period falls in with that of cortical changes underlying membrane separation. In the egg of *Nereis* the period also falls in with the period of profound cortical changes. These should now be described.

The cortical changes in the egg of *Nereis* subsequent to the at-

tachment of the spermatozoön have been amply described by Lillie (1911, 1912). Lillie's observations have been confirmed by Packard, Dungay, and Just (1912). Similar changes take place in the egg of *Platynereis* (Just, 1915*a*). Now Kite, at Lillie's request, removed the spermatozoön from the inseminated egg soon after attachment (cf. also Goodrich), showing that such removal as that by centrifugal force (Lillie, 1912) does not interfere with the cortical changes. Mere attachment of the sperm, therefore, suffices for cortical changes.

In the eggs of both *Nereis* and *Platynereis* the sperm remains external to the egg for several minutes, though, as I have pointed out (Just, 1915*c*), Wilson has erroneously figured the sperm *within* the egg (!) during the stage of rupture of the germinal vesicle (Wilson, *The Cell in Development and Inheritance*, 1896, p. 141, Fig. 71A; and 1900, p. 191, Fig. 97A). This is clearly a case of mistaken identity. In *Nereis* the sperm reaches the stage figured by Wilson at the end of first maturation. (Cf. also Wilson's erroneous figures—Fig. 71B, *loc. cit.*, 1896, p. 141, and Fig. 97B, *loc. cit.*, 1900, p. 191—of the sperm and its aster at the early anaphase of first maturation instead of at the prophase of the second.)[1]

During part of this period of sperm attachment the egg undergoes profound cortical changes. First, the cortex, is emptied of its jelly, leaving what are probably the walls of the cortical alveoli which as strands cross the perivitelline space, their outer ends inclosed by a delicate membrane, which is undoubtedly the plasma membrane, lying beneath the vitelline membrane. Thus, the cortical layer is more sharply defined. Next, the egg exhibits amoeboid changes, during which it darkens and contracts, the perivitelline space narrowing; the sperm is discerned with difficulty. These changes ensue during the 25-minute period immediately following insemination. The egg now clears and rounds up, the perivitelline space widening; the sperm is again clearly in evidence external to the egg. The period of susceptibility to distilled water coincides exactly with this period of cortical changes. Thus, the egg of Nereis resembles that of *Echinarachnius* in its response to treatment with dilute sea-water.

[1] Wilson in his cell lineage of *Nereis* confused the eggs of *Nereis* and of *Platynereis*.

It would thus seem that the water-holding capacity of the egg of *Nereis* is in part at least due to the integrity of the cortex. Breakdown of the cortex renders the egg easily accessible to the destructive action of distilled water. In the uninseminated egg, cytolysis does not occur until the cortex breaks down, as shown by loss of jelly; then the egg is without adequate protection. In the inseminated egg, susceptibility comes on during the period that the egg is in a critical state, because of structural changes in the cortex. Susceptibility here again is correlated with cortical state. Fundamentally, therefore, there is no difference between the egg of *Echinarachnius* and that of *Nereis* (and of *Platynereis*).

These observations are important not only for the analysis of the cortical changes induced by insemination but also for an analysis of the mechanism of water-holding by living cells. It is from these two points of view that I wish now to discuss the work here reported.

DISCUSSION

Owing largely to the influence of Loeb's work on experimental parthenogenesis, it has been generally assumed that the separation of the vitelline membrane ("formation" of the so-called "fertilization membrane") is fertilization. I have been at pains to show that in echinid ova where this separation of the vitelline membrane is a most distinctive response to insemination this is *not* what I have called the fertilization-reaction. In the first place, in ova of both *Arbacia* and *Echinarachnius* definite cortical changes precede those responsible for the separation of the membrane; also, the block to polyspermy intervenes before this membrane separation (Just, 1919*a*). Secondly, the analysis of the action of butyric-acid sea-water (Just, 1919*c*, 1920) and that of hypertonic sea-water (Just, 1922*d*, 1922*e*) show that in experimental parthenogenesis, quite apart from the fact that experimental parthenogenesis is not fertilization, the mechanism of membrane separation is not at all that postulated by Loeb.

If anything were wanting further to strengthen this position of mine, the observations herein described would supply that want. For if the cortical changes underlying membrane separation in the ova of *Arbacia* and *Echinarachnius* be the fertilization reaction, then

the comparable but far longer enduring cortical changes in *Nereis* and *Platynereis* ova constitute the fertilization reaction. It would therefore be logical to conclude that the duration of the fertilization reaction varies widely with different ova. This might be. But to me there is a distinct gain in bringing the fertilization reaction into the category of other biological reactions, capable of time-measurement. True, one may set up any definition of fertilization that one wishes to set up. And this indeed has been done. Thus, Tennent, for example, says:

> Usually we have thought of fertilization as comprising the whole series of changes occurring during the time extending from the instant of contact of the spermatozoön with the surface of the egg to the completion of fusion of the germ nuclei, although we have tacitly admitted the logic of the position that fertilization is not completed until the conjugation of homologous maternal and paternal chromosomes. In practise, however, the word fertilization is often used as though it applied merely to the initial impulse to development.

Definitions like this render it well nigh impossible adequately to measure time limits of the fertilization process and thus to make its physiology easily comparable to muscle contraction, nerve conduction, etc. The kinetics of fertilization is rendered extremely unlikely in terms of such definitions.

Cortical changes of membrane separation and jelly extrusion per se, as I see them, are not the fertilization reaction; these changes differ in different ova, and yet in all these there is a fundamental cortical change due to the immediate effect of sperm attachment. This is practically instantaneous. Membrane separation and jelly extrusion are evidences of cortical breakdown. Moreover, they are of interest for their own sake quite apart from the fertilization-reaction. They are the final explosion of a series of blaze-like cortical reactions induced by insemination. That the cortical changes incident to membrane separation in echinid ova and those incident and subsequent to jelly extrusion in nereid ova are fundamentally the same, the experiments on the effects of hypotony on the latter here reported clearly show.

But these experiments are noteworthy from an entirely different point of view. The brutal method of exposing eggs to distilled water results in cytolysis. Such cytolysis has been described by others not

only with distilled water but by other agents. The experiments also show that in less dilute sea-water the uninseminated egg remains viable; this is true of the inseminated egg also. What I wish here to emphasize is that the response of the eggs to extreme dilutions of sea-water is totally unlike that to less dilute sea-water. Interesting as the phenomena of cytolysis are, they are of less significance for the study of hydration and dehydration than the changes in *viable cells capable of complete reversal when restored to their normal medium from the hypo- and hyper-tonic solutions that do not injure.* It is in such solutions that we must seek the analysis of the mechanism of the water-holding power of viable cells.

SUMMARY

1. The uninseminated egg of *Nereis* breaks down in distilled water in 120–240 seconds. This cytolysis moves as a wave over the egg after the initial effect of the dilute sea-water in separating the vitelline membrane.

2. Less dilute sea-water does not cause cytolysis as rapidly as distilled water. Thus, in 20 per cent sea-water a majority of the eggs remain intact for hours.

3. For 25 minutes after insemination eggs of *Nereis* remain highly susceptible to distilled water, as shown by the rate of cytolysis. This is the period of cortical changes.

4. Eggs of *Platynereis* respond to exposure to extreme dilutions of sea-water in much the same may.

5. The experiments suggest that the cortical reactions are not the fertilization reaction. They are also important as a basis of comparison with effects of less dilute sea-water later to be reported.

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